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Alpha-Synuclein Expression Influences the Processing of the Amyloid Precursor Protein

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Alpha-Synuclein Expression Influences the Processing of the Amyloid Precursor Protein

Hazel Laura Roberts

A thesis submitted for the degree of Doctor of Philosophy

University of Bath
Department of Biology & Biochemistry

October 2016

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LIST OF ACRONYMS AND ABBREVIATIONS

AD	Alzheimer's disease
ADAM10/17	A disintegrin and metalloproteinase-10/ -17
AICD	APP intracellular domain
AP1	Activator protein-1
APLP1/2	Amyloid precursor-like protein-1/ -2
APP/ sAPP	Amyloid precursor protein/ soluble APP
Aβ	β -Amyloid
BACE1	Beta-site APP cleaving enzyme-1
CHIP	C-terminus of Hsc70 Interacting Protein
CMA	Chaperone-mediated autophagy
DLB	Dementia with Lewy bodies
EO-FAD	Early onset familial Alzheimer's disease
ER	Endoplasmic reticulum
GGA3	Golgi-localized, gamma adaptin ear-containing, ARF-binding-3
iPSC	Induced pluripotent stem cell
IRE	Iron-responsive element (of mRNA)
JNK1	Jun kinase-1
LOAD	Late onset Alzheimer's disease
LTP/LTD	Long term potentiation/ long term depression
MAM	Mitochondrial-associated membrane (of ER)
MSA	Multiple system atrophy
NAC	Non-amyloid component region of α -synuclein
NF-κB	Nuclear factor- κ B
PD/ PDD	Parkinson's disease/ Parkinson's disease dementia
PERK	PKR-like ER kinase
PKC	Protein kinase C
PKR	Protein kinase R
PS1/2	Presenilin-1/-2
ROS	Reactive oxygen species
SNARE	SNAP (soluble NSF attachment protein) receptor
SNpc	Substantia nigra pars compacta
syn	(α -/ β -/ γ -) Synuclein
TGN	Trans-Golgi network

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The best sea: has yet to be crossed.

The best child: has yet to be born.

The best days: have yet to be lived;

and the best word that I wanted to say to you

is the word that I have not yet said.

- Nasim Hikmet, translated by Richard McKane

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ABSTRACT

In certain neurodegenerative diseases such as Dementia with Lewy Bodies (DLB), it is hypothesised that misfolded α -synuclein (α -syn) and β -amyloid both contribute to pathology. α -Syn and β -amyloid have been suggested to synergistically promote one another's accumulation and aggregation, but the mechanisms are unknown. β -Amyloid is generated from β -/ γ -secretase-mediated processing of the amyloid precursor protein (APP). This study investigated how α -syn overexpression in cells affects β -amyloid production from APP, using multiplex assays, luciferase reporter assays, and western blotting. Wildtype α -syn expression induces β -amyloid generation from APP in SH-SY5Y human neuroblastoma cells, and similar changes to APP processing occur in another neuronal cell model. Dominant-negative overexpression of α -syn mutants revealed that disrupting the N-terminal domain can increase APP amyloidogenic processing. Secretase enzymes that perform APP processing were next investigated. γ -Secretase activity, measured by a luciferase reporter, was not increased by α -syn overexpression. A higher ratio of β - to α -secretase processing was hypothesised, which led to expression and activity studies of the major β - and α -secretases, BACE1 and ADAM10 respectively. It was shown that the BACE1 protein expression is post-transcriptionally upregulated in α -syn cells, with increased APP cleavage in cells. ADAM10 protein expression is transcriptionally suppressed in wild-type α -syn cells, reducing total levels of catalytically active enzyme. However the change in ADAM10-mediated APP processing may be negligible since, critically, plasma membrane expression of ADAM10 appears to be maintained. To aid understanding of the mechanism that connects α -syn to APP processing, BACE1 expression was used in pharmacological studies of cell stress signalling. This approach revealed that in α -syn cells BACE1 lysosomal and/or proteasomal degradation may be disturbed. Additionally, BACE1 expression is induced by translational de-repression mediated by eIF2 α ser-51 phosphorylation, which was increased in α -syn cells. Although preliminary, the data suggests a role for oxidative stress mediating the increased BACE1 expression in wild-type α -syn cells.

CHAPTER 1: INTRODUCTION

1.1 Synucleinopathies

1.1.1 Parkinson's disease and other synucleinopathies

A 'synucleinopathy' is a neurodegenerative disease where the primary pathology is misfolding of the protein α -synuclein (α -syn). Parkinson's disease (PD) is the most common synucleinopathy, and affects approximately 140 people per 100,000 in the UK (Wales et al. 2013; Wickremaratchi et al. 2009). PD is also known as a 'Lewy body disease', since the most visible results of α -syn misfolding are Lewy bodies. 'Lewy bodies' are globular inclusions of insoluble aggregated proteins, primarily α -syn fibrils, in the cell bodies of neurons (Spillantini et al. 1997). The term 'Lewy neurite' was coined for a spindle-like neurite inclusion, pictured along with Lewy bodies in Figure 1.1 (Braak et al. 2003). PD is diagnosed when a patient exhibits characteristic levodopa-responsive motor and autonomic symptoms ('parkinsonism'), with no dementia, and autopsy reveals Lewy bodies and selective loss of dopaminergic neurons (Berg et al. 2014).

The other synucleinopathies are Parkinson's disease dementia (PDD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). PDD and DLB patients are characterised by parkinsonism and Lewy bodies, like PD, but are additionally defined by dementia symptoms. Presentation of dementia more than a year after a PD diagnosis leads to a classification of PDD. DLB patients are diagnosed with dementia before, or within a year, of emerging parkinsonism (Lippa et al. 2007; McKeith et al. 1996). MSA patients exhibit parkinsonism or cerebellar ataxia, a condition where balance and co-ordination are impaired. A conclusive diagnosis can only be made with post-mortem histology, since MSA is defined by predominantly oligodendroglial inclusions of α -syn, in addition to some neuronal Lewy bodies (Gilman et al. 2008; Peelaerts & Baekelandt 2016).

Alzheimer's disease is not considered to be a synucleinopathy, despite the frequent presence of Lewy bodies in AD brains. Since AD brains have widespread insoluble aggregates of other neurodegenerative disease proteins, Lewy bodies are not the major pathology. Furthermore, Lewy bodies in AD tend to be confined to the amygdala (Hamilton 2000; Parkkinen et al. 2003; Jellinger 2004).

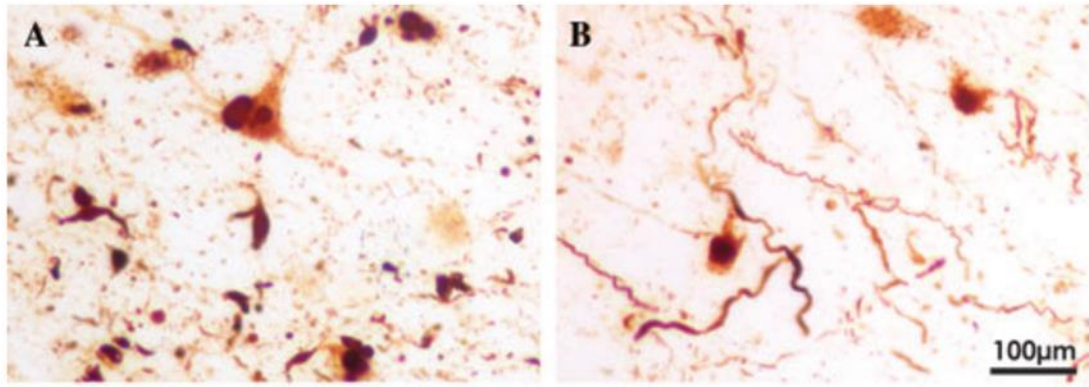


Figure 1.1 Images of α -syn-immunopositive inclusions, in the SNpc of PD brains. (A) Lewy bodies, (B) Lewy neurites. Taken from (Halliday & McCann 2010).

1.1.2 A brief history of Parkinson's disease

James Parkinson's 'An Essay on the Shaking Palsy' in 1817 is considered to be the seminal work on what would later be termed Parkinson's disease, describing the natural history of the disease rather than merely symptoms. Lewy Bodies were identified in 1912 by Fritz Heinrich Lewy, and in 1919 Konstantin Tretiakoff found that the key anatomical feature of PD is severe lesioning of the substantia nigra pars compacta (SNpc). It took decades for the significance of selective SNpc damage to be understood as the root cause of motor symptoms (Lees 2007). The discovery of the neurotransmitter dopamine by Arvid Carlsson in the 1950s was key. Depleted dopamine levels in the caudate and putamen of PD brains were discovered by Hornykiewicz and colleagues in 1960. This was followed by discovery of dopaminergic neurons that connect the SNpc to the striatum, and that damage to the SNpc reduces dopamine levels in the striatum. Thus the biochemical basis of PD was understood well enough for the symptoms of disease to be treated, using new drugs to improve dopaminergic transmission (Fahn 2008). The root cause of neurodegeneration was a mystery until a genetic connection was uncovered. A large family of Italian descent was found to have a 'PD gene' with 85% penetrance, in the chromosomal region 4q21–q23. The gene was identified in 1997 as SNCA, coding for α -syn, with an A53T point mutation (Polymeropoulos et al. 1997). In the same year, α -syn was discovered to be a major insoluble component of Lewy Bodies by Spillantini and colleagues (Spillantini et al. 1997). Spillantini et al. predicted that the A53T mutation promotes α -syn aggregation into insoluble α -syn fibrils, confirmed a year later (Conway et al. 1998). From these discoveries, a new field of research into the role of α -syn in PD emerged. Nevertheless, other genetic causes of familial PD have also subsequently been identified: Parkin (1998), DJ-1 (2001), PINK1 (2004), LRRK2 (2004), PLA2G6 (2009) and VPS35 (2011). Mutation of these genes can result in PD-type symptoms, but also some atypical characteristics (Houlden & Singleton 2012).

1.1.3 A ‘spectrum’ of diseases containing Lewy Bodies

There is an ongoing debate about whether all forms of genetic and sporadic PD share a common mode of pathogenesis, triggered by defects in an interconnected network, or whether there are multiple unrelated diseases under the umbrella of ‘PD’ (Houlden & Singleton 2012). Furthermore, researchers have puzzled over the relationship between PD, the Lewy body dementias, and AD. On the one hand, PDD and DLB are widely accepted to be related to PD (Lippa et al. 2007). The genetics are similar: SNCA and LRRK2 mutations appear to manifest as PD, PDD, or DLB (Poulopoulos et al. 2012). The neuropathology also suggests common origins: diagnosis of PDD/DLB corresponds with the widespread presence of cortical Lewy bodies, in addition to brainstem Lewy bodies. Cortical Lewy bodies have been suggested to originate from the brainstem (Irwin et al. 2013). Lewy body pathology appears to progress along specific neuronal pathways, in a stereotyped manner from the brainstem to the neocortex, as characterised by Braak et al. (Figure 1.2) (Braak et al. 2003). Cell-to-cell transmission has also been experimentally demonstrated (Walker et al. 2015; Desplats et al. 2009). On the other hand, some researchers advocate that DLB is equally related to AD as PD. Genetic risk factors studied by genome-wide array show that DLB shares about the same number of genetic determinants with AD as with PD (Guerreiro et al. 2015). This was supported by an independent genetic study, focussing on only major AD and PD genes in samples from PDD/DLB patients. A number of mutations and copy-number variants were found in both AD-related genes (APP, PSEN1, PSEN2, MAPT) and PD-genes (SNCA, LRRK2, PARK2) (Meeus et al. 2012). The genetic evidence clearly supports mechanistic overlaps between PD, Lewy body dementias, and AD. Neuropathological evidence also suggests a complex picture, for example some DLB brains present little or no cortical Lewy body pathology (Irwin et al. 2012; Pletnikova et al. 2005). Furthermore, cognitive impairment in PDD/DLB appears to correlate with the appearance of pathologies typical of AD: intracellular tau tangles and extracellular β -amyloid plaques (Irwin et al. 2013).

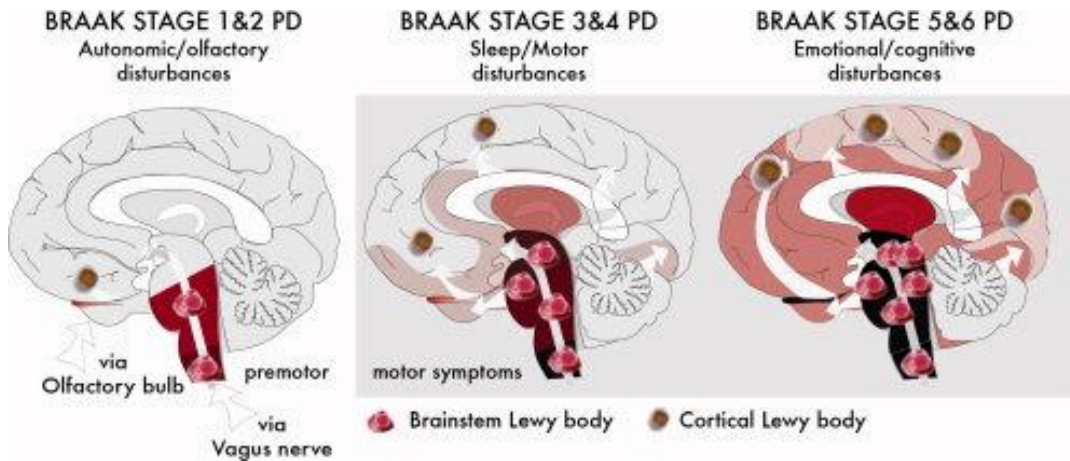


Figure 1.2 Illustration of Braak staging. Lewy bodies are hypothesised to spread in a caudal to rostral direction, from the brainstem to neocortex. Taken from (Halliday et al. 2011).

1.1.4 The toxic oligomer hypothesis

α -Syn-containing insoluble inclusions are the diagnostic feature of Lewy body diseases (Berg et al. 2014), and familial point mutations linked with familial PD appear to increase aggregation of the protein *in vitro* (Conway et al. 2000; Li et al. 2001). This naturally led to the idea that α -syn aggregates are toxic. Soluble β -sheet-rich oligomers are now widely regarded to be the major toxic species of α -syn, a theory known as ‘the toxic oligomer hypothesis’. The toxic oligomer hypothesis is not unique to α -syn, in fact other proteins and peptides such as $A\beta_{42}$, a peptide associated with Alzheimer’s disease (AD), can form β -sheet-rich oligomers that are toxic to cells (Kayed et al. 2003; Kaye et al. 2004). The feature that these amyloidogenic proteins have in common is an intrinsically-disordered monomer, which can explore a range of conformational states in solution, including conformations with β -strands (Uversky 2009). The current model for their formation of amyloid fibrils is one of nucleation followed by polymerisation, with a likely structural-conversion step of the oligomeric intermediates in between (Figure 1.3) (Cremades et al. 2012; Jarrett & Lansbury 1992). Under the right conditions, such as agitation at room temperature, the proteins spontaneously nucleate small globular oligomers with a core of antiparallel β -strands. These oligomers appear to have the ability to permeabilise membranes (Cremades et al. 2012; Kaye et al. 2004; Lorenzen et al. 2014; Lashuel et al. 2002; Celej et al. 2012; Kostka et al. 2008; Danzer et al. 2007). Small oligomers can grow by monomer addition. Additionally a slow conformational change can compact the oligomers into highly structured β -sheet forms, which are proteinase-K resistant, appear to be more toxic, and produce high levels of ROS (Cremades et al. 2012; Iljina et al. 2016). The compact oligomers polymerise into amyloid fibrils, with each additional monomer folding into the parallel β -sheet conformation by templating.

Amyloid fibrils can de-polymerise when monomer concentration is low, or may break into fragments that nucleate several new fibrils (Cremades et al. 2012; Knowles et al. 2009).

α -Syn can oligomerise in its wild-type state, particularly if the local concentration of monomers is increased, but the process is faster with PD familial point mutations such as E46K (Li et al. 2001; Conway et al. 2000; Conway et al. 1998). E46K causes only very subtle changes to the monomeric conformation of α -syn, and does not reduce the long-range interactions between N- and C-terminus that protect against fibrillisation (Fredenburg et al. 2007; Bertoni et al. 2005; Breydo et al. 2012; Rospigliosi et al. 2009). However, E46K does disrupt the formation of small α -helical oligomers, increasing the concentration of disordered monomeric protein in the cytosol (Dettmer et al. 2015). There is evidence to support multiple toxic effects of α -syn toxic oligomers to cells, which is illustrated in Figure 1.4 and has been reviewed (Roberts & Brown 2015). It is potentially helpful to consider the features common to all toxic oligomers, to reveal where toxicity originates. For instance, $A\beta_{42}$ aggregates have a close correlation between their toxicity and affinity for 1-anilinonaphthalene 8-sulfonate (ANS). ANS binds exposed hydrophobic regions in partially unfolded proteins, and responds by fluorescing more brightly. It reveals that toxic oligomers of $A\beta$, and a number of other amyloidogenic oligomers, expose hydrophobic patches (Bolognesi et al. 2010). Furthermore, the hydrophobic exposure of toxic oligomers could be linked to the ease at which they appear to permeabilise membranes. Campioni et al. demonstrated this link using the bacterial protein HypF-N as a model. HypF-N reliably forms two types of morphologically similar β -sheet-rich oligomer, one of which is toxic and the other benign. The toxic form had a greater tendency to bind ANS, less hydrophobic packing of residues, and appeared to permeabilise SH-SY5Y cells to calcium influx when added externally (Campioni et al. 2010). The specific ability of α -syn oligomers to permeabilise membranes has also been characterised in detail by multiple groups (Lorenzen et al. 2014; van Rooijen et al. 2009; Danzer et al. 2007; Kostka et al. 2008).

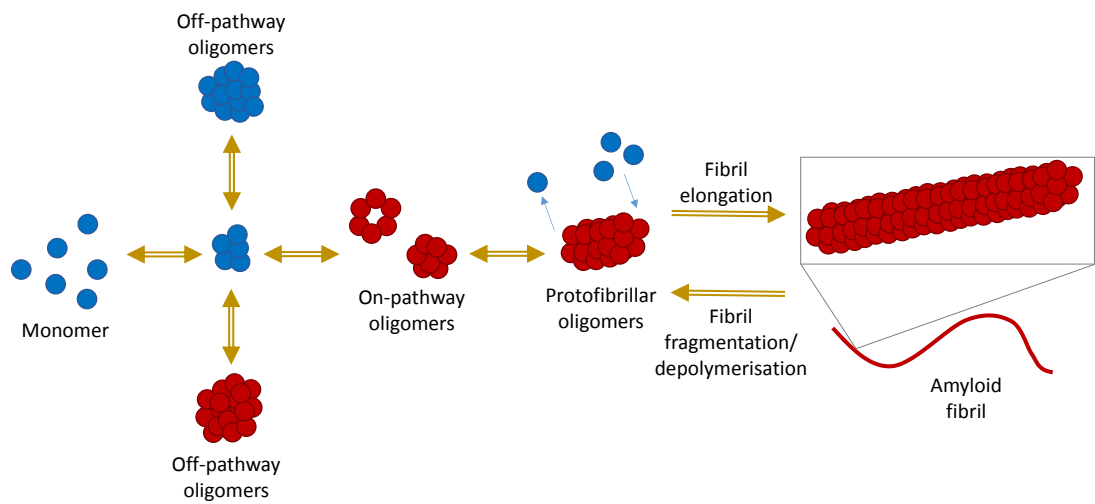


Figure 1.3 Model schematic of α -syn oligomerisation and amyloid fibril formation. ‘Off-pathway’ α -syn oligomers can form with little or no β -sheet secondary structure (coloured blue), or ‘on-pathway’ oligomers with antiparallel β -structure (coloured red). A structural conversion event creates protofibrils with parallel β -structure, which elongate by monomer addition into mature amyloid fibrils. Adapted from (Roberts & Brown 2015).

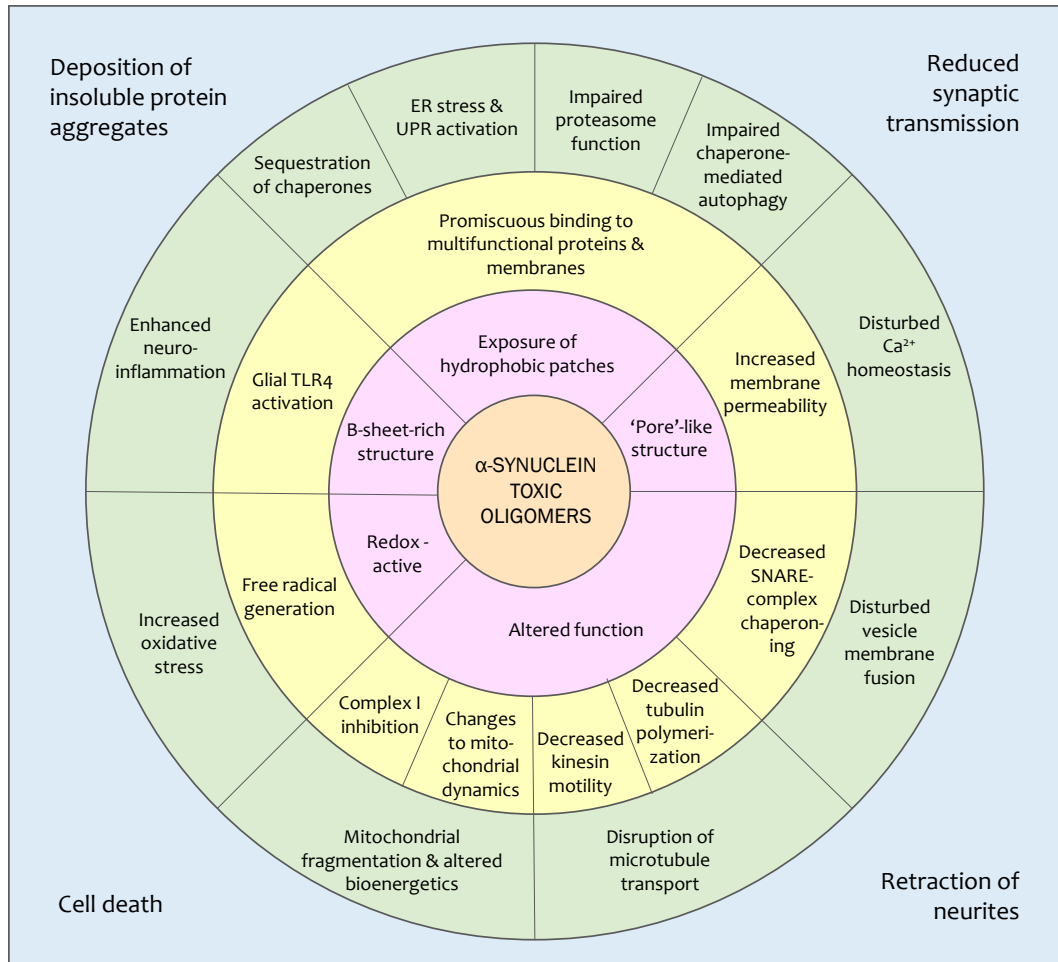


Figure 1.4 Effects of toxic oligomers. Diagram summarising the proposed links between cell processes that are disturbed by α -synuclein toxic oligomers (outer rings), and the properties of oligomers (inner ring). ER: endoplasmic reticulum. UPR: unfolded protein response. Adapted from (Roberts & Brown 2015).

1.1.5 Genetic and environmental factors converge to promote α -syn aggregation

The pathogenic aggregation of α -syn may arise from a combination of genetic and environmental factors. One genetic factor is SNCA, which can cause PD through pathogenic point mutations or copy number variants. Up to 10% of PD is familial, and Mendelian inheritance of α -syn copy number variants comprises about 2% of familial PD (Lesage & Brice 2009). Some sporadic cases of α -syn gene multiplication have also been detected, and a 'Rep1' nucleotide polymorphism close to the SNCA promoter is a validated risk factor for sporadic PD (Lesage & Brice 2009; Maraganore et al. 2006). Since α -syn multiplications and mutations are uncommon, the presence of α -syn aggregates in the majority of PD cases needs explanation. One potential reason is the presence of other monogenic loci that affect α -syn accumulation. LRRK2 is the most common, with its missense variants accounting for up to 10% of familial PD and 3.6% of sporadic PD. The G2385R and R1628P variants in Asian populations confer a 2-3-fold increased susceptibility for PD (Lesage & Brice 2009). LRRK2 appears to have an important role in autophagy and lysosomal function that makes it vital for the normal degradation of α -syn (Gan-Or et al. 2015). The G2019S mutation, common to North African and Ashkenazi Jew PD patients (Lesage & Brice 2009), has been shown to inhibit chaperone-mediated autophagy (CMA) and promote accumulation of α -syn, a CMA substrate (Orenstein et al. 2013). Several other familial PD genes, and hits from genome-wide association studies, appear to converge on autophagy pathways. This suggests that α -syn accumulation is frequently downstream of lysosomal dysfunction in PD (Gan-Or et al. 2015).

Environmental factors and the intrinsic processes of aging are likely to also contribute to sporadic PD, and promote downstream α -syn accumulation. Exposure to certain pesticides, toxins, and particular metals such as manganese, are linked to increased risk of PD. Proteasome inhibition, induced by the pesticides paraquat and maneb, and oxidative stress, caused by rotenone or MPTP through respiratory chain inhibition, have been experimentally shown to promote α -syn aggregation (Burbulla & Krüger 2011). Neuronal oxidative stress and respiratory chain inhibition are also reported in cases of manganese toxicity (Martinez-Finley et al. 2013). Manganese exposure causes frontal cortex neurodegeneration in primates, and reduced function of nigrostriatal neurons. Curiously, this is accompanied by both intracellular α -syn aggregates and diffuse extracellular A β aggregates, presumably due to the sensitivity of both amyloidogenic proteins to oxidative stress (Verina et al. 2013). The aging process itself also appears to lead to selective SNpc α -syn accumulation in healthy humans and primates (Chu & Kordower 2007). Aging reduces proteasome and autophagic activity, and also leads to defective mitochondria not being degraded and replaced efficiently, increasing oxidative stress. α -Syn appears to participate in 'vicious cycles' whereby it exacerbates these deficits,

as illustrated in Figure 1.5, and forms more toxic species as a result. However, the specific vulnerability of the SNpc to α -syn accumulation and neurodegeneration is peculiar, particularly given that this phenomenon does not occur in rodents (Bobela et al. 2015). Bolam and Pissadaki hypothesise that it is the unusually high energy demands of SNpc dopaminergic neurons in humans and primates, caused by their architecture and lack of myelination, which makes them more sensitive to oxidative stress and mitochondrial dysfunction (Bolam & Pissadaki 2012; Bobela et al. 2015).

1.1.6 Is α -syn an essential driver of Lewy Body disease?

α -Syn misfolding is a common denominator in familial and sporadic PD. However, the extent to which α -syn drives neurodegeneration is debatable. At one extreme, McGeer & McGeer propose an ‘ α -syn burden hypothesis’, whereby sporadic PD entirely results from “a declining ability to clear α -syn” (McGeer & McGeer 2008). At the other extreme, it is argued that α -syn-independent mechanisms of neurodegeneration exist in some types of PD. Evidence for this stems experimentally from animal models of rare genetic forms of PD, involving loss-of-function of GBA1 or ATP13A2. These genetic models experience degeneration of the SNpc even when crossed with an α -syn-null background (Keatinge et al. 2015; Kett et al. 2015). Furthermore, patients with PARK2 mutations exhibit a juvenile-onset recessive form of PD, where Lewy bodies are generally absent (Houlden & Singleton 2012). PARK2 specifically regulates mitophagy, demonstrating that mitochondrial dysregulation may be sufficient to cause a subtype of PD (Gan-Or et al. 2015). Nonetheless, α -syn-independent neurodegeneration in PD is rare, and proves only that PD is a heterogeneous disorder.

A moderate view is perhaps more widely accepted, whereby α -syn has a major role in driving the progression of neurodegeneration in PD, but other genetic and cell factors are also important, as discussed in Section 1.1.5. The experimental evidence that α -syn aggregation is toxic to cells is robust. Numerous studies have shown that recombinant oligomers of α -syn decrease the viability of cell cultures to which they are added, and that A53T, E46K, and H50Q disease-associated point mutations enhance both the rate of aggregation and toxicity

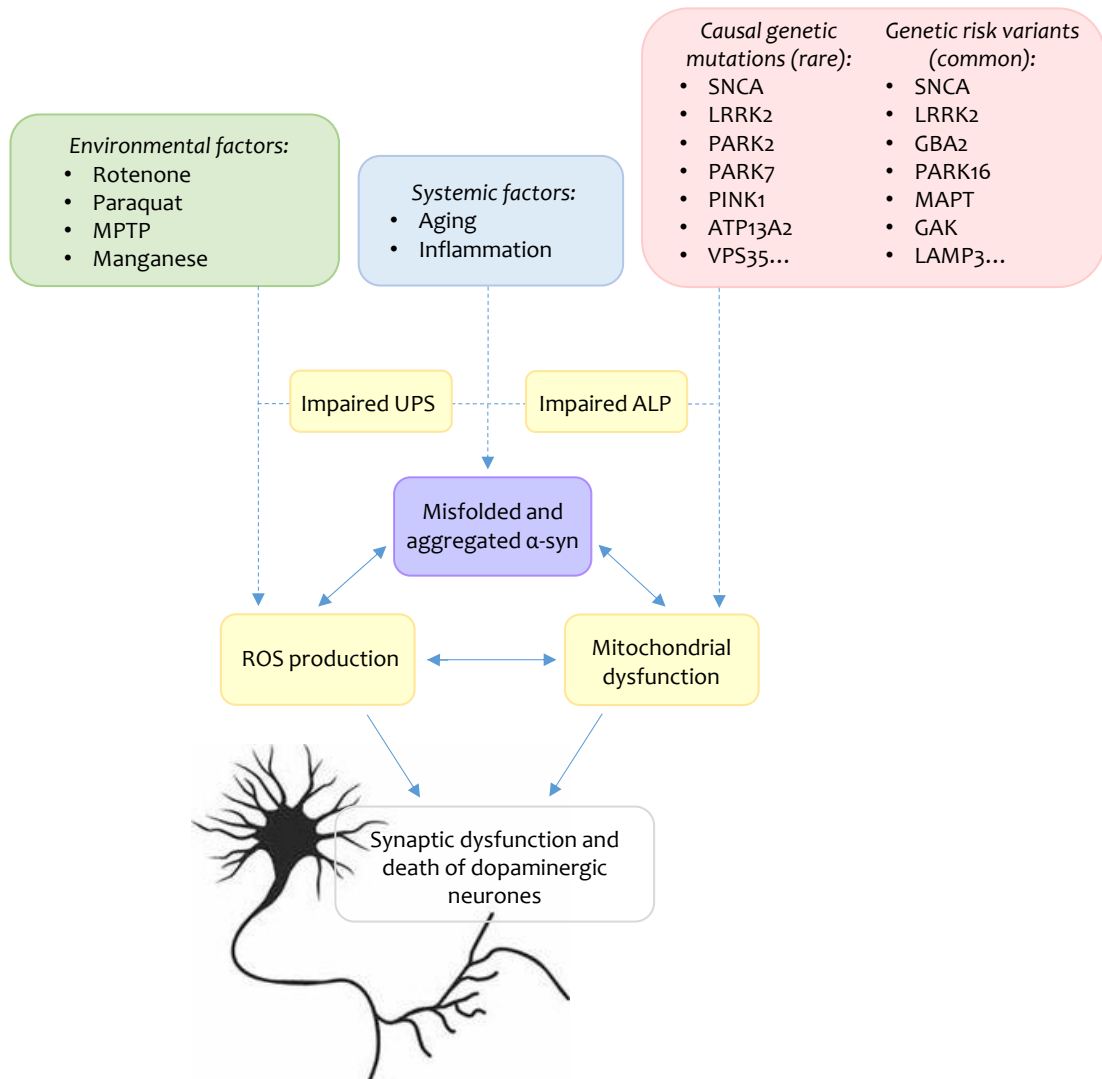


Figure 1.5 Simplified schematic showing the proposed convergence of environmental, systemic, and genetic factors in Lewy body disease. Mitochondrial dysfunction and ROS production are two of the major factors that lead to dopaminergic cell death, but are not the only toxic effects of α -syn, which include ER stress and altered Ca^{2+} signalling. Environmental/systemic/genetic factors influence the aggregation of α -syn, in part, through impairing the ubiquitin-proteasome system (UPS) or autophagy-lysosome pathway (ALP) (Houlden & Singleton 2012). α -Syn aggregation furthermore participates in a ‘vicious circle’ that increases mitochondrial dysfunction and ROS production.

(Choi et al. 2004; Khalaf et al. 2014; Conway et al. 1998; Pandey et al. 2006). Pharmacological inhibition of α -syn aggregation, using baicalein, protected E46K α -syn-treated cells from proteasome block and mitochondrial depolarisation (Li et al. 2011). To test the hypothetical toxicity of α -syn oligomers a ‘conformation-trapped’ E57K α -syn protein, which promotes the formation of β -sheet-rich oligomers but strongly inhibits fibrillisation, was developed by structure-based design. Overexpression of E57K α -syn increased the calcium influx and reduced the viability of cell cultures. Moreover, severe dopaminergic neuron loss was seen in rats injected with E57K α -syn lentivirus, more pronounced than demonstrated with E46K or wild-type α -syn (Winner et al. 2011). This evidence supports a leading role for α -syn toxic oligomers in PD.

There is also strong evidence that α -syn aggregates can be transmitted from cell to cell, propagating disease (Costanzo & Zurzolo 2013). Native secretion and endocytosis of α -syn were demonstrated with conditioned media taken from cell lines. α -Syn in conditioned media was endocytosed by primary cortical neurons and promoted cell death. This was prevented by either upregulating HSP70 chaperone expression, immunodepleting α -syn from conditioned media, or treating conditioned media with oligomer-interfering compounds (Danzer et al. 2011; Emmanouilidou, Melachroinou, et al. 2010). ‘Seeding’ of endogenous α -syn aggregation, by applying exogenous α -syn fibril fragments, was also demonstrated within cells, and labelled α -syn transmitted between cells in co-cultures (Volpicelli-Daley et al. 2012; Desplats et al. 2009; Hansen et al. 2011). The kinetics of seeding α -syn aggregation was described in detail by Iljina et al., who suggest that templated seeding is inefficient by itself, but aggregation is strongly aided by the production of ROS by soluble oligomers, illustrated in Figure 1.6 (Iljina et al. 2016). *In vivo* studies also support the propagation of α -syn aggregates, (a) in healthy non-transgenic dopaminergic neurons grafted into α -syn transgenic rodents (Hansen et al. 2011; Angot et al. 2012); and (b) in neurons of healthy transgenic/wild-type mice, following injection of recombinant fibrils (Luk, V. M. Kehm, et al. 2012; Luk, V. Kehm, et al. 2012). Compellingly, the injection of α -syn fibrils into non-transgenic mice appears sufficient to trigger a PD-like cascade of Lewy pathology transmission, between anatomically connected regions. Selective dopaminergic neuron loss from the SNpc, sparing the adjacent ventral tegmental area, was also observed and is a classic feature of PD (Luk, V. Kehm, et al. 2012). Therefore, seeding and propagation experiments support a role for α -syn in driving the progression of Lewy Body disease.

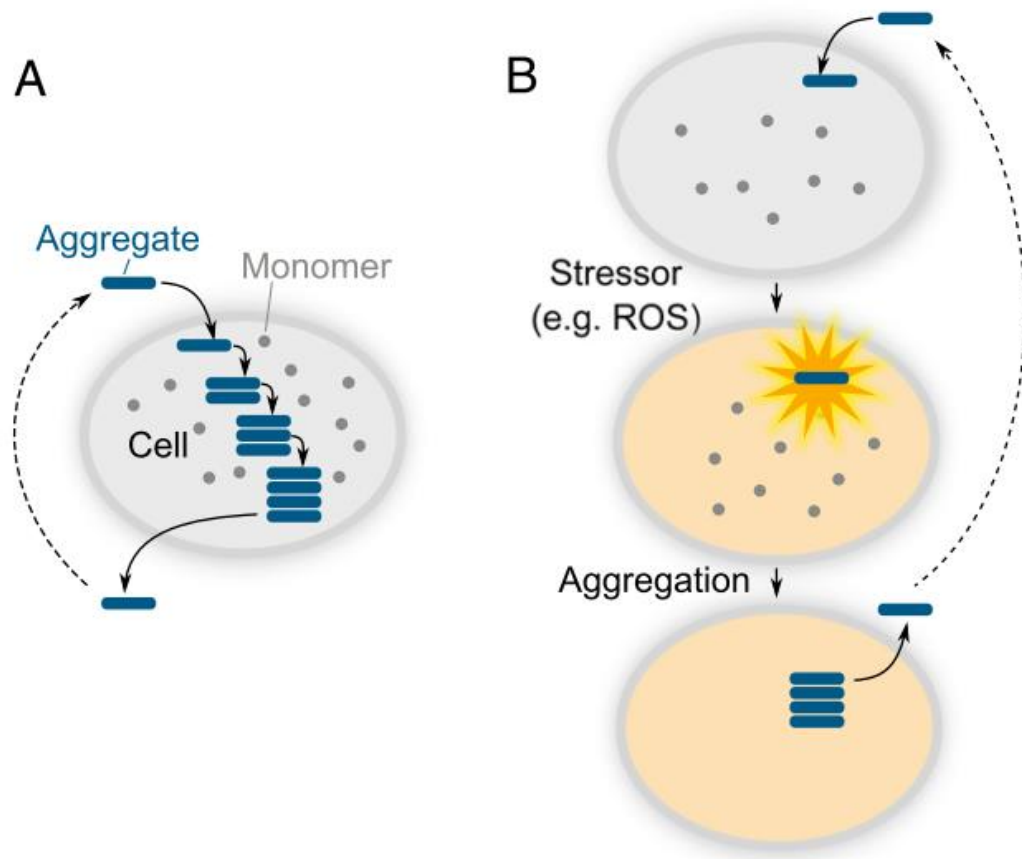


Figure 1.6 Two simplified models of α -syn aggregate propagation in Lewy body disease. Templated seeding of aggregates, a ‘prion-like’ style of propagation where exogenous aggregates are grown by addition of endogenous monomers; **(B)** Cell-driven aggregation, where exogenous aggregates induce cell stress, creating conditions that indirectly promote endogenous α -syn aggregation. Taken from (Iljina et al. 2016).

1.2 Structure and localisation of α -syn

1.2.1 The synuclein family of proteins

Synuclein was first identified in 1988, in the electric organ of the electric ray *Torpedo californica*, and was named for its apparent localisation to both synaptic vesicles and the nuclear membrane (Maroteaux et al. 1988). Interestingly, α -syn is still considered to have an important role at pre-synaptic vesicles, but its presence and function in the nucleus is contentious (Wales et al. 2013). Synuclein was then independently discovered as a brain-specific bovine protein (1990), a protein involved in synaptic plasticity in songbirds (1995), and precursor of the ‘non amyloid component’ (NAC) peptide (1993), abundant in amyloid plaques of AD patients (Clayton & George 1998; Uéda et al. 1993; George et al. 1995; Nakajo et al. 1990). The NAC precursor was later given the name α -synuclein, and its homologs β -synuclein and γ -synuclein identified as having similar sequences to the bovine protein and *Torpedo* protein respectively (Jakes et al. 1994; Clayton & George 1998).

α -Syn, β -syn, and γ -syn have genes at chromosome sites 4q21, 5q35 and 10q23, respectively (Wales et al. 2013). A schematic comparison of the synuclein proteins can be seen in Figure 1.7. The N-terminal 42 amino acids for the three proteins are highly homologous, and β -syn shares 61% of its amino acid sequence with α -syn, differing mainly in the C-terminal half (Wales et al. 2013; Jakes et al. 1994). Both α - and β -syn are predominantly expressed in the brain, particularly in presynaptic terminals of the frontal cortex, striatum and hippocampus (Iwai et al. 1995). α -Syn also appears to be important for the development of lymphocyte cells (Shameli et al. 2015). γ -Syn is less closely related to α -syn, and expressed most abundantly in the cell bodies and axons of the peripheral nervous system, as well as in brain neurons (Buchman et al. 1998).

Interestingly the hydrophobic domain of β -syn, which corresponds to the amyloid fibril-forming ‘NAC’ region of α -syn, is missing a stretch of 11 amino acids. (Jakes et al. 1994). *In vitro* and *in vivo* experiments appear to show that β -syn is more resistant to aggregation than α -syn, and even inhibits the fibrillisation of α -syn (Fan et al. 2006; Hashimoto et al. 2001). Yet all three synucleins can form fibrils *in vitro*, although β - and γ -syn require specific conditions (Yamin et al. 2005; Uversky et al. 2002). All three synucleins form neuronal inclusions when over-expressed in mice (Taschenberger et al. 2013; Ninkina et al. 2009), and appear to form aggregates in PD and DLB brains (Surgucheva et al. 2014; Galvin et al. 1999). As previously discussed, mutations and gene copy variants of α -syn are associated with familial Lewy Body disease. Mutations of β -syn, V70M and P123H, have also been discovered

in patients with DLB (Ohtake et al. 2004), and a single nucleotide polymorphism of the γ -syn gene confers a significant risk for DLB (Nishioka et al. 2010).

1.2.2 Disordered monomers and α -helical tetramers

α -Syn is an intrinsically-disordered protein, which means that it lacks a definitive secondary or tertiary structure. In solution, α -syn exists in a range of conformations. The acidic C-terminus is entirely unfolded, and the hydrophobic NAC region exists in a compact ‘molten globule’ state. The traditional view of α -syn in a cell is that the majority is cytosolic, and therefore an unstructured monomer (Breydo et al. 2012). Yet metastable α -helical tetramers were reported in 2012, using non-denaturing gels or *in vivo* cross-linking to prevent their dissociation (Bartels et al. 2012). In normal human brains there appears to be twice as many tetramers as monomers, depending on the antibody used. Tetramers are assumed to be native and non-toxic, given their prevalence in healthy tissues (Dettmer et al. 2015). The issue provoked much debate, with publications from various groups either confirming (Bartels et al. 2012; Dettmer et al. 2013; Gould et al. 2014; Dettmer et al. 2015) or contesting the predominance of tetramers (Fauvet et al. 2012; Lashuel et al. 2013). Tetramers have achieved more credence for their potential to explain a common mechanism by which familial PD-associated point mutations affect α -syn. The tetramer:monomer ratio is significantly decreased by A30P, E46K, H50Q, G51D, and A53T, relative to the wild-type protein over-expressed in cells. Furthermore, additional N-terminal domain E \rightarrow K substitutions were created in the E46K construct, which caused stepwise decreases in the tetramer:monomer ratio and reduced cell viability (Dettmer et al. 2015). This demonstrates that tetramers are disrupted by PD-associated point mutations, confirming previous modelling (Kara et al. 2013; W. Wang et al. 2011), and that disruption is potentially cytotoxic. The current working hypothesis is that a reduction in tetramer formation increases the monomer concentration in the cell, leading to greater opportunity for pathological β -sheet rich oligomers to form (Kara et al. 2013; Dettmer et al. 2015).

1.2.3 Membrane-binding and sub-cellular localisation of α -syn

In addition being cytosolic, up to a third of cellular α -syn binds to membranes as a monomer or multimer (Visanji et al. 2011). Membrane-binding imposes some secondary structure upon α -syn. Spanning the first 95 residues of α -syn are seven 11-residue repeats, with striking resemblance to the amphipathic α -helices found in apolipoproteins (Davidson et al. 1998). Upon membrane-binding, the first 100 residues of α -syn become α -helical, whereas the acidic C-terminal 101-140 residues remain unstructured (Eliezer et al. 2001). NMR analysis of α -syn binding to SDS micelles originally suggested a two-helix model (Bussell & Eliezer 2003).

Subsequent EPR and FRET experiments have shown that α -syn can also bind to unilamellar vesicles as a single unbroken helix (Figure 1.8), these having less extreme curvature than SDS micelles (Alderson & Markley 2013; Jao et al. 2008).

α -Syn exclusively binds lipids with acidic headgroups, and appears to favour vesicles of smaller diameter (20–25 nm) as opposed to larger (~125 nm) vesicles (Davidson et al. 1998). The ability of α -syn to sense high curvatures, and membranes with particular lipid compositions, has been suggested to contribute to its sub-cellular localisation (Middleton & Rhoades 2010). α -Syn localises to pre-synaptic vesicles, which have high curvature and a moderate negative charge (George et al. 1995; Snead & Eliezer 2014; Maroteaux et al. 1988). Mitochondrial membranes are another site where α -syn has been detected, potentially due to their curvature and the preference of α -syn for mitochondrial lipid cardiolipin (Snead & Eliezer 2014; Nakamura et al. 2011). The mitochondrial dysfunction evident in α -syn transgenic models may partly stem from α -syn inner or outer membrane localisation (Nakamura 2013). In conjunction with inner mitochondrial membrane localisation, α -syn has been shown to specifically and dose-dependently inhibit complex I activity in some models (Devi et al. 2008; Loeb et al. 2010; Liu et al. 2009; Chinta et al. 2010), although not in others (Sarafian et al. 2013; Kamp et al. 2010; Nakamura et al. 2011). The outer membrane localisation of over-expressed α -syn has been shown to promote mitochondrial fragmentation, through direct alteration of the properties of the membrane (Kamp et al. 2010; Nakamura et al. 2011). Another, recently discovered, location enriched in α -syn is the sites of ER-mitochondria contact, within the specialised ER domain that is rich in anionic phospholipids and cholesterol (Calì et al. 2012; Guardia-Laguarta et al. 2014).

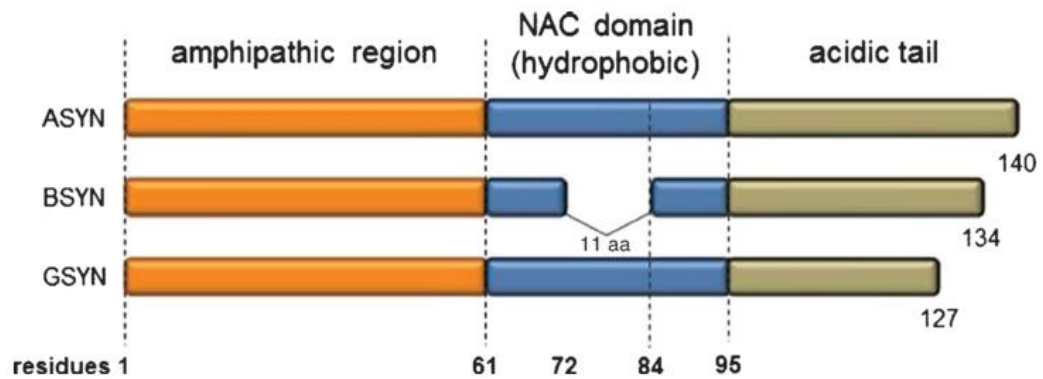


Figure 1.7 Schematic comparison of the synuclein proteins. α -Syn (ASYN), β -syn (BSYN), and γ -syn (GSYN) have high homology in the N-terminal amphipathic region, but differ in the structure of the acidic C-terminal tail. β -Syn is also missing a section of 11 amino acids contained in the NAC domain of α -syn (Taken from Wales et al. 2013).

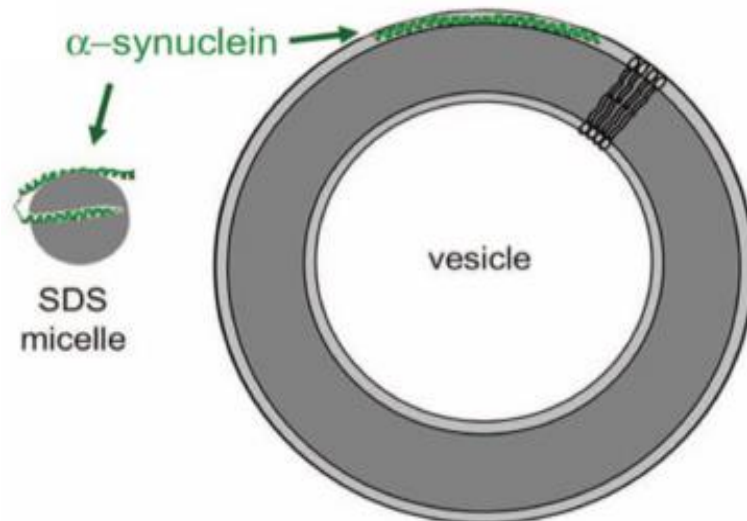


Figure 1.8 Model of 9-89 α -syn in a two-helix and extended helix conformation. The extended helix is more common in the presence of SUVs, but highly curved micelles cannot accommodate a long extended helix. Adapted from (Alderson & Markley 2013).

1.3 Function of α -syn

1.3.1 Vesicle docking and fusion

The propensity of α -syn to bind pre-synaptic vesicles was described when it was first identified (Iwai et al. 1995; George et al. 1995). Functional studies of α -syn have frequently centred on synaptic vesicle regulation. No obvious phenotype results from single or double α -/ β -syn knockout in mouse models (Chandra et al. 2004). A triple α -/ β -/ γ -syn knockout mouse model had reduced striatal dopamine levels, but displayed ‘hyperdopaminergic’ behaviours, and striatal dopamine release under electrical stimulation was enhanced (Anwar et al. 2011). Conversely, overexpression of α -syn decreases neurotransmitter release from PC12 and primary adrenal cells, corresponding with an accumulation of docked synaptic vesicles in the presynaptic terminal (Larsen et al. 2006).

A large number of roles for α -syn in regulating synaptic vesicles have been proposed, including vesicle clustering, docking, fusion, and recycling. Figure 1.9 illustrates a few hypotheses (Snead & Eliezer 2014). The following summarises two of the key theories about the effects of α -syn on vesicle docking and fusion. One prevailing hypothesis is the ‘SNARE complex chaperone’ theory. Burré et al. demonstrated that membrane-bound α -syn, potentially as α -helical oligomers, promote the assembly of trans-SNARE complexes that mediate synaptic vesicle fusion with the plasma membrane (Burré et al. 2010; Burré et al. 2014). α -Syn dose-dependently promoted SNARE complex assembly in HEK293T cells, and also primary neurons from synuclein knock-out mice. Synaptobrevin-2, a v-SNARE, was shown to bind the C-terminus of α -syn, and C-terminal truncated α -syn was incompetent at promoting SNARE complex assembly (Burré et al. 2010). Clustering of synthetic synaptic vesicles by α -syn also appears to depend on the presence of synaptobrevin-2 (Diao et al. 2013). Despite its involvement in SNARE complex assembly, α -syn does not appear to actively promote exocytosis in cells. Choi et al. suggest that the role of α -syn is to keep the cis-SNARE complex stable in the plasma membrane, rather than directly regulating trans-SNARE complexes (Choi et al. 2013).

In addition to exocytosis, a similar involvement of α -syn with SNAREs has been studied in the early secretory pathway between the ER and Golgi. A53T α -syn was found to directly interact with membrin and syntaxin-5 of the ER- and Golgi-SNAREs by Thayanidhi et al. (Thayanidhi et al. 2010). Formation of ER/Golgi trans-SNARE complexes was inhibited by A53T mutant α -syn, and ER-Golgi transport delayed (Thayanidhi et al. 2010). Delayed ER-Golgi transport is not unique to the mutant α -syn protein. Several mammalian cell models overexpressing wild-type α -syn, at physiologically relevant levels, display reduced ER-Golgi

trafficking (Oaks et al. 2013; Thayanidhi et al. 2010). Since wild-type α -syn does not appear to impair exocytosis (Choi et al. 2013), there are clearly differences in the behaviour of α -syn at different membranes. The origin of these differences have been suggested to arise from either the protein-binding partners of α -syn (Thayanidhi et al. 2010), or distinct lipid compositions of the membrane affecting the oligomeric state of α -syn (Wang & Hay 2015). These possibilities remain to be explored.

The ‘SNARE complex chaperone’ theory of α -syn function is not universally accepted. Contradictory evidence exists that suggests α -syn directly inhibits vesicle docking/fusion, without acting on other proteins (Snead & Eliezer 2014). Some groups did not find that α -syn physically interacts with SNARE proteins, or promotes SNARE complex formation (DeWitt & Rhoades 2013; Darios et al. 2010). Kamp et al. additionally demonstrated that α -syn can inhibit vesicle fusion *in vitro* using protein-free assays, and only the membrane-bound N-terminal portion of α -syn is necessary for the effect. A suggested model for the reduced vesicle fusion involved α -syn stabilising membrane lipid packing defects (Figure 1.10), which relieves curvature stress (Kamp et al. 2010; Braun & Sachs 2015). Lai et al. were in agreement, using a more complete proteoliposome fusion assay including SNAREs. C-terminal truncated α -syn inhibited vesicle fusion as much as the wild-type, which excludes a direct interaction with synaptobrevin-2. Additionally, replacement of synaptobrevin-2 with a distantly-related yeast SNARE had no effect on the ability of α -syn to inhibit vesicle fusion (Lai et al. 2014).

1.3.2 Membrane curvature

The inhibitory effect of α -syn on *in vitro* vesicle fusion assays, discussed above, reveals that α -syn can profoundly influence the stability of curved membranes. A suggested function of α -syn is to create and maintain membrane curvature (Bendor et al. 2013). When added to solutions of artificial vesicles, α -syn can create networks of membrane tubules, with structures resembling budding vesicles. The structures were noted to be similar to the effects of specialised curvature-inducing proteins such as amphiphysin (Bendor et al. 2013; Jao et al. 2008; Varkey et al. 2010). At a molecular level, α -syn binds via a curved amphipathic helix to small unilamellar vesicles. Shallow insertion of hydrophobic residues appears to reduce surface tension and increase the positive pressure of core hydrocarbon chains, resulting in membrane undulations (Braun & Sachs 2015).

As yet there is only limited evidence that supports the membrane curvature-inducing properties of α -syn *in vivo*. α -Syn localises to outer mitochondrial membranes and/or ER mitochondrial-associated membranes (MAM), both of which have high curvature and contain

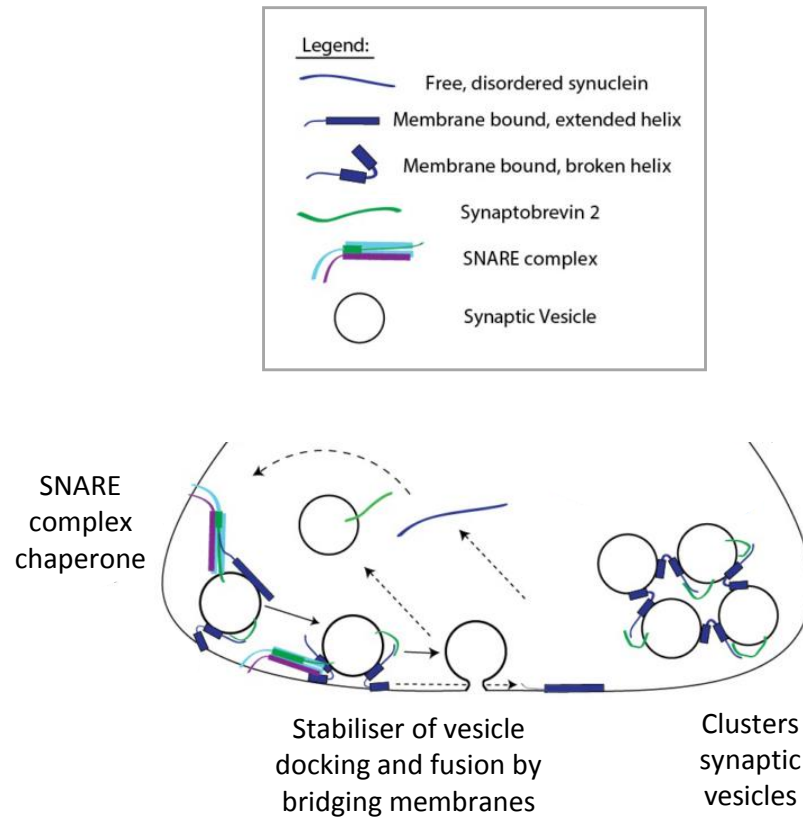


Figure 1.9 Three potential effects of α -syn on synaptic vesicles. α -Syn may promote SNARE complex assembly, act as a physical bridge between the vesicle and plasma membrane, or cluster together synaptic vesicles. Adapted from (Snead & Eliezer 2014).

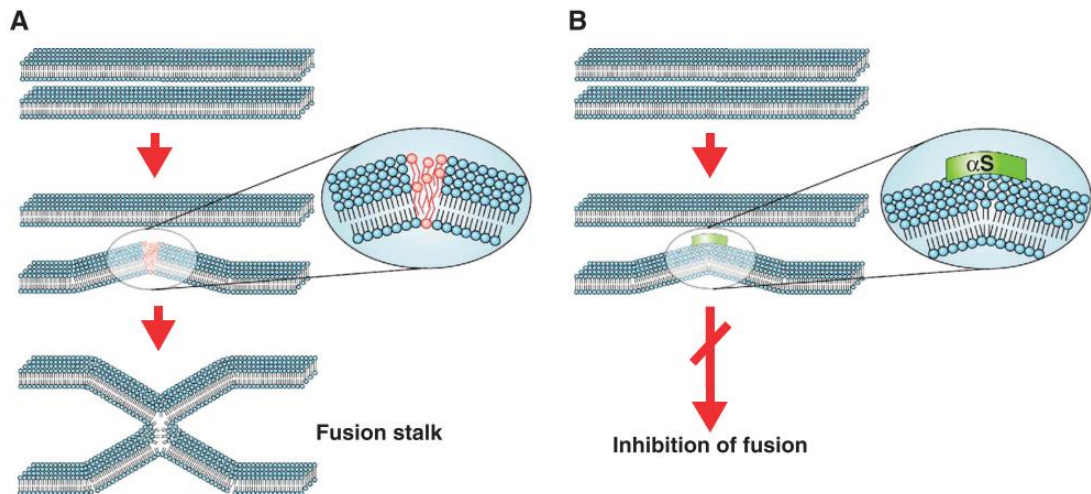


Figure 1.10 Model for a direct SNARE-independent effect of α -syn on vesicle fusion. Fusion of two adjacent membranes requires defects in lipid packing that allow the membranes to be pinched into a fusion stalk. **(B)** α -Syn stabilises lipid packing defects and may impede fusion by this route. Taken from (Kamp et al. 2010).

high levels of anionic phospholipids (Kamp et al. 2010; Guardia-Laguarta et al. 2014). Overexpression of α -syn dramatically remodels the mitochondrial network in cultured cells and primary neurons. Mitochondrial fragmentation appears to be triggered by α -syn overexpression, and can occur even in the absence of mitochondrial fission protein Drp1 (Kamp et al. 2010; Nakamura et al. 2011). α -Syn also appears to promote formation of MAM projections from the ER, and enhances calcium transfer from ER to mitochondria (Cali et al. 2012; Guardia-Laguarta et al. 2014). These observations could be indicative of wider cellular functions for α -syn membrane-binding and membrane-remodelling, beyond the synaptic vesicle fusion paradigm.

1.3.3 Iron regulation

α -Syn is a redox-active metal-binding protein, with a site in the extreme N-terminus that complexes Cu (II), and two sites in the C-terminus that can bind Fe (III) (Rasia et al. 2005; Davies, Moualla, et al. 2011; Davies, Wang, et al. 2011; Bharathi & Rao 2007; Binolfi et al. 2006). One interesting proposed function for α -syn, although currently unexplored *in vivo*, is as a cellular ferrireductase (Davies, Moualla, et al. 2011; Brown 2013). Ferrireductase activity of α -syn has been demonstrated *in vitro*, and appears to depend on the presence of copper as a cofactor. Cell lines overexpressing α -syn contain elevated cytosolic levels of Fe(II) relative to Fe(III) (Davies, Moualla, et al. 2011). A role for α -syn in maintaining cellular iron homeostasis is also suggested by the iron-dependent translational control of α -syn. A putative iron regulatory element has been identified in the α -syn 5'UTR, and iron chelation reduces α -syn mRNA levels (Febbraro et al. 2012; Friedlich et al. 2007). It is not yet clear whether the iron-reducing activity of α -syn is associated with its function at membranes. Unpublished work from our laboratory suggests that the ferrireductase activity of α -syn occurs in a membrane-bound fraction, so the two functions could be linked (McDowall & Brown, unpublished).

1.4 Alzheimer's disease

1.4.1 A historical overview of Alzheimer's disease

Alzheimer's disease (AD) is named after Alois Alzheimer, who wrote a seminal case study in 1907 of a woman with severe dementia, describing senile plaques and neurofibrillary tangles found in the cortex upon autopsy. This was by no means the first time that such connections had been made: a few months previously neurofibrillary tangles had been linked with dementia by S. C. Fuller, and senile plaques had been described in dementia as far back as 1887 (Ramirez-Bermudez 2012). Neurofibrillary tangles have since been characterised as intra-neuronal inclusions containing insoluble fibrillar aggregates of the tau protein, a microtubule

regulator (Kosik et al. 1988; Goedert et al. 1989). Extracellular senile plaques, otherwise known as ‘amyloid plaques’, were shown to be largely composed of fibrillar β -amyloid fragments from the amyloid precursor protein (APP), first cloned in 1987 by three independent groups (Goldgaber et al. 1987; Kang et al. 1987; Tanzi et al. 1987). As mentioned previously, the NAC fragment of α -syn would be subsequently identified as the second most common component of amyloid plaques (Uéda et al. 1993). Yet by the early 1990s it seemed clear that β -amyloid deposition is the central event of AD, leading to a cascade of pathology including neurofibrillary tangles (Hardy & Allsop 1991). This idea was developed and refined into the ‘amyloid cascade hypothesis’ (Hardy & Higgins 1992).

In 1991 the first mutation responsible for early onset familial AD (EO-FAD) was sequenced, within the APP gene itself (Goate et al. 1991). Subsequently, mutations in the presenilin-1 and presenilin-2 genes (PSEN1/2) were found to be more common in EO-FAD (Tanzi 2012). Presenilins are essential for the proteolytic cleavage of APP to form β -amyloid, and pathogenic mutations can alter the location of cleavage (De Strooper et al. 1999; Wolfe et al. 1999; Tanzi 2012). Since then, almost all of the >200 mutations reported in APP, PSEN1, and PSEN2 for EO-FAD have proved to be autosomal-dominant and fully-penetrant. Most increase the tendency of β -amyloid to aggregate, by enhancing the production of the A β 42 variant or by amino acid substitutions within the β -amyloid sequence. The ‘Swedish’ APP mutation, and also duplications of the APP gene, increase production of all β -amyloid species (Tanzi 2012). Strong links between β -amyloid dysregulation and EO-FAD support the amyloid cascade hypothesis.

Late-onset AD (LOAD), defined as occurring after the age of 65, appears to have a heritability of 60-80% (Bergem et al. 1997; Gatz et al. 1997; Pedersen et al. 2001). Yet LOAD has not been associated with variants of APP or presenilins, and is considered to be multifactorial, which has been used as a criticism of the amyloid cascade hypothesis (Tanzi 2012; Harrison & Owen 2016). In 1993 a variant of the apolipoprotein E gene, ApoE- ϵ 4, was found to be overrepresented in LOAD patients compared with age-matched controls (Strittmatter et al. 1993). The instigation of genome wide association studies (GWAS) for AD risk factors, from 2007 onwards, allowed other common risk variants to be discovered that are present in over 5% of the population. Yet the newly-discovered risk variants, save for TREM2, only weakly increase AD risk (Tanzi 2012; Reiman et al. 2007). TREM2 (Triggering Receptor Expressed on Myeloid cells 2) is a microglial surface receptor, and has been shown to bind to ApoE- ϵ 4. The two proteins may be central to a pathway that promotes microglial phagocytosis of apoptotic neurons (Atagi et al. 2015). Other GWAS hits include genes that are proposed to have roles in β -amyloid production and clearance, or alter lipid metabolism, cellular signalling,

or innate immunity (Tanzi 2012). Rare mutations are excluded from GWAS, but may also significantly contribute to the heritability of LOAD. For example, in 2009 two rare loss-of-function mutations were identified in ADAM10, which is an enzyme that cleaves APP through the middle of the β -amyloid sequence (Kim et al. 2009). The complex genetics and environmental influences on LOAD may therefore be reconcilable with the amyloid cascade hypothesis, if changes in β -amyloid are a common denominator.

1.4.2 Current state of the amyloid cascade hypothesis

The amyloid cascade hypothesis is still widely accepted, although modified over the years to focus on ‘toxic oligomers’ of β -amyloid as a disease-causing agent. Yet scepticism has grown in response to the failures of γ -secretase inhibitors, and immunotherapy against β -amyloid oligomers, to arrest the progression of dementia in clinical trials, with a minority renouncing the amyloid cascade hypothesis (Drachman 2014; Castello & Soriano 2014). Alternative theories for the cause of sporadic AD include impaired microvasculature integrity in the brain (Drachman 2014), or neuroinflammation causing cerebral insulin resistance (Clark et al. 2012). Researchers still in favour of the amyloid cascade hypothesis see clinical failures as resulting from poor trial design, or evidence that β -amyloid accumulation is an early trigger of a disease process that becomes self-sustaining (Harrison & Owen 2016; Mullane & Williams 2013). Currently there is insufficient reason to abandon the amyloid cascade hypothesis, but its future may depend on the success of amyloid-altering drug treatments in pre-clinical AD patients.

1.4.3 Toxic oligomers of β -amyloid

Amyloid plaques, consisting of extracellular linear fibrils of β -amyloid, are surrounded by dystrophic neurites and activated glia in the brain, and appear to have a direct involvement in neuronal death (Hardy & Allsop 1991). However, numbers of amyloid plaques do not correlate with the severity of AD, and can be detected in brains, on average, about ten years before the average age of clinical AD onset (Perrin et al. 2009). Thus the hypothesised toxicity of β -amyloid in the aforementioned ‘amyloid cascade hypothesis’ is unlikely to stem from amyloid plaques.

AD is now considered to be a disease of synaptic dysfunction. Synapse loss correlates strongly with cognitive decline in AD patients, appearing to have a greater effect on cognitive decline than neuron death (Terry et al. 1991). In the 1990s soluble oligomeric species of β -amyloid were identified, and Lambert et al. discovered that these could inhibit LTP in organotypic CNS cultures, in the absence of fibrils (Lambert et al. 1998; Ferreira et al. 2015).

Rodent models confirmed the propensity of β -amyloid oligomers to impair synapse function, causing memory impairment (Ferreira et al. 2015). Memory impairments in mice with a mutant APP transgene were shown to be easily reversible, by suppression of the transgene, despite the persistence of amyloid deposits (Fowler et al. 2014). Similarly, in humans β -amyloid oligomers have been linked to memory impairment. AD patients were shown by Bjorklund et al. to have β -amyloid oligomers localised hippocampal cell fractions enriched for the post-synaptic density marker PSD-95. However in cognitively normal individuals that exhibited some amyloid plaques and neurofibrillary tangles, β -amyloid oligomers were absent from hippocampal post-synaptic fractions, despite their confirmed presence in the brain (Bjorklund et al. 2012).

β -amyloid oligomers have a multitude of cellular effects that may promote synaptic dysfunction (Ferreira et al. 2015). In addition to changes to synaptic plasticity-related receptors (Roselli et al. 2005; Hsieh et al. 2006), β -amyloid oligomers appear to cause increased oxidative stress (De Felice et al. 2007), ER stress (Ma et al. 2013; Yoon et al. 2012), calcium release from intracellular stores (Zempel et al. 2010; Paula-Lima et al. 2011), and also defects in axonal transport that may be triggered by calcineurin activation (Ramser et al. 2013).

1.5 The amyloid precursor protein

1.5.1 Structure and localisation of APP

APP is a type I integral membrane protein with a large, extracellular, N-terminal domain and small, cytoplasmic, C-terminal domain, illustrated in Figure 1.11. The extracellular domain consists of an E1 domain, and E2 domain, followed by the A β sequence that partly runs into the membrane-spanning region. E1 encompasses a heparin-binding/growth factor-like domain (HFBD/GFLD), and sub-domains for copper- and zinc- binding. A second HFBD/GFLD is found in the E2 domain. Within the cytoplasmic domain is a YENPTY protein-interaction motif (Jacobsen and Iverfeldt 2009). APP has two homologs, APLP1 and APLP2, with high conservation of the YENPTY, E1 and E2 motifs, which may be responsible for their apparent redundancy of function *in vivo*. Interestingly, the A β sequence is exclusive to APP (Zheng & Koo 2011). The A β sequence is present in three of the major mRNA splice isoforms of APP: APP695, APP751, and APP770. APP751 and APP770 are expressed ubiquitously, whereas APP695 is confined to neurons and has the highest neuronal expression (Tanaka et al. 1989).

After synthesis in the ER, APP is transported the Golgi to the trans-Golgi network (TGN), undergoing phosphorylation and glycosylation along the way. APP is highly abundant in the

TGN, due to retention. From there, APP is trafficked to the plasma membrane, and can either be ‘secreted’ by α -secretase cleavage of the extracellular domain, or internalised by clathrin-mediated endocytosis. Endosomal APP is recycled to the plasma membrane or TGN, or transported to lysosomes for degradation (Jiang et al. 2014). Delivery of APP to the plasma membrane results in localisation with the post-synaptic density, however APP is also detected in pre-synaptic vesicles (Del Prete et al. 2014; Hoey et al. 2009).

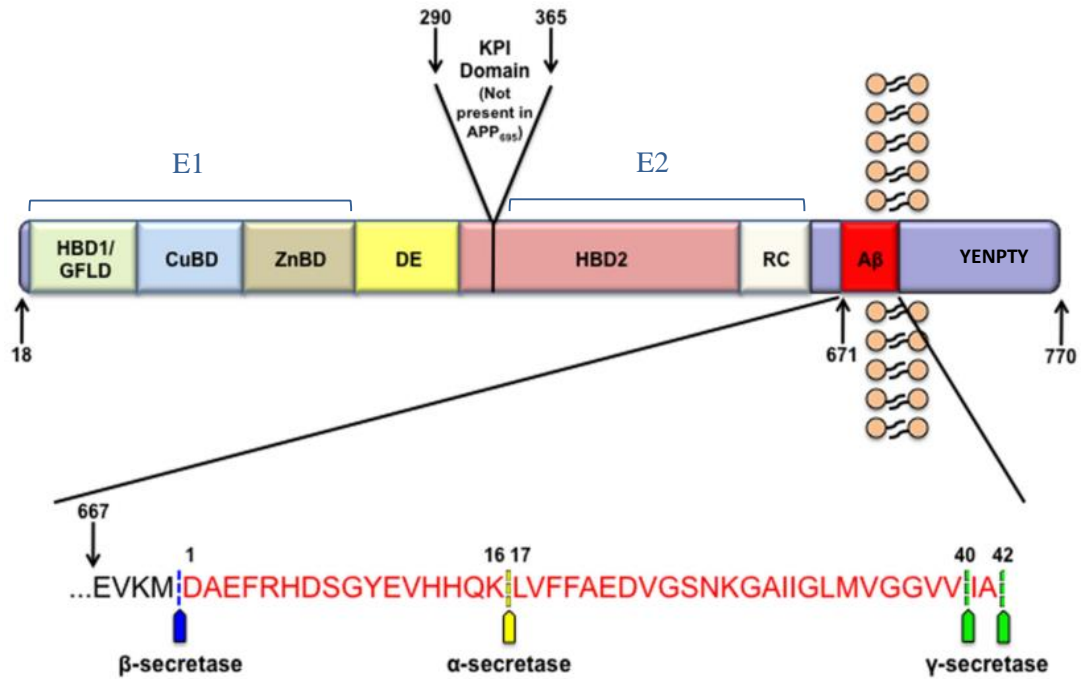


Figure 1.11 Schematic of APP695, with amino acid numbering based on the full-length 770 sequence. APP695 is a splice variant missing the Kunitz-type protease inhibitor domain (KPI). From the N-terminus there is a heparin-binding/growth factor-like domain (HFBD/GFLD), a copper-binding domain (CuBD), a zinc-binding domain (ZNBD), and acidic region (DE), a second heparin-binding domain (HFBD2), a random coil region (RC), an amyloid- β domain (A β), and a YENPTY protein-interaction motif at the C-terminus. The A β sequence is inset in red, illustrating the typical cleavage sites of α -, β -, and γ -secretase (Adapted from Lazarov & Demars 2012).

1.5.2 Secretase-mediated processing of APP

Cleavage of APP by α -, β -, and γ -secretases can occur at any point in the secretory/endocytic pathway after APP has been O-glycosylated in the Golgi (Tomita et al. 1998). Sites include the TGN, synaptic vesicles, plasma membrane, and endosomes, shown in Figure 1.12 (Del Prete et al. 2014; Jiang et al. 2014). α -Secretase cleavage of APP occurs largely at the plasma membrane and TGN (Parvathy et al. 1999; Skovronsky et al. 2000),

whereas β -, and γ -secretases tend to localise to lipid rafts in endosomes and the TGN, and are most active in acidified late endosomes (Ehehalt et al. 2003; Vetrivel et al. 2004). Thus changes to the transport or retention of APP in various subcellular compartments can have consequences for secretase cleavage (Jiang et al. 2014).

The β -secretase is β -site APP cleaving enzyme 1 (BACE1), a transmembrane aspartic protease, and can cleave APP at Asp1 and Glu11 of the A β domain (Vassar et al. 1999). γ -Secretase is a large complex of presenilin (PS1/ PS2), the catalytic domain; nicastrin, the scaffold; anterior pharynx defective-1 (APH-1), and presenilin enhancer-2 (PEN2), both regulatory subunits (Kimberly et al. 2003; Takasugi et al. 2003; Wolfe et al. 1999). γ -Secretase cleaves the other, C-terminal, end of the A β sequence at multiple sites that produce several lengths of A β species from 38-43 amino acids. A β 40 is the most common product *in vivo*, but A β 42 is more closely linked to AD pathogenesis (Zhang et al. 2012). Several FAD mutations to the APP or PS1 genes do not enhance A β production significantly, or even reduce γ -secretase activity, but cause γ -secretase to favour cleavage that generates A β 42 (Scheuner et al. 1996). Even in sporadic AD, A β 42 appears to be selectively deposited in amyloid plaques. A β 42 is more hydrophobic than other β -amyloids, has a higher tendency to generate fibrils than A β 40 *in vitro*, and forms soluble oligomers that appear more toxic to cells (Snyder et al. 1994; El-Agnaf et al. 2000; Burdick et al. 1992). Three other cleavage products are formed in the amyloidogenic pathway from combined β -/ γ -secretase activity, illustrated in Figure 1.13 (Fodero-Tavoletti et al. 2011). β -Secretase cleavage yields an N-terminal 'sAPP β ' domain, released into the extracellular or intraluminal space, and on the C-terminal side the β C-terminal fragment (β CTF), a 99-amino acid peptide also known as 'C99', remains in the membrane. C99 is then cleaved by γ -secretase to release the transmembrane A β peptide, and the C-terminal APP intracellular domain (AICD) (Zhang et al. 2012).

A further layer of complexity is added by the parallel activity of α -secretase, which partially competes with β -secretase for cleavage of the full-length APP (Skovronsky et al. 2000; Colombo et al. 2013). Combined α -/ γ -secretase activity on APP is generally referred to as the non-amyloidogenic pathway (Zhang et al. 2012). The transmembrane 'a disintegrin and metalloproteases', ADAM10 and ADAM17, have α -secretase activity. ADAM10 is essential for constitutive α -secretase cleavage of APP in neurons, and both ADAM10 and ADAM17 appear to contribute to activity-stimulated α -secretase cleavage of APP (Kuhn et al. 2010; Marcello et al. 2007). In the Golgi network, the ADAM10 proenzyme has an autoinhibitory pro-domain, which is cleaved by furin during the secretory pathway (Lammich et al. 1999). ADAM10 activity is further regulated by increased trafficking to the plasma membrane and decreased exocytosis during LTP (Marcello et al. 2007; Marcello et al. 2013).

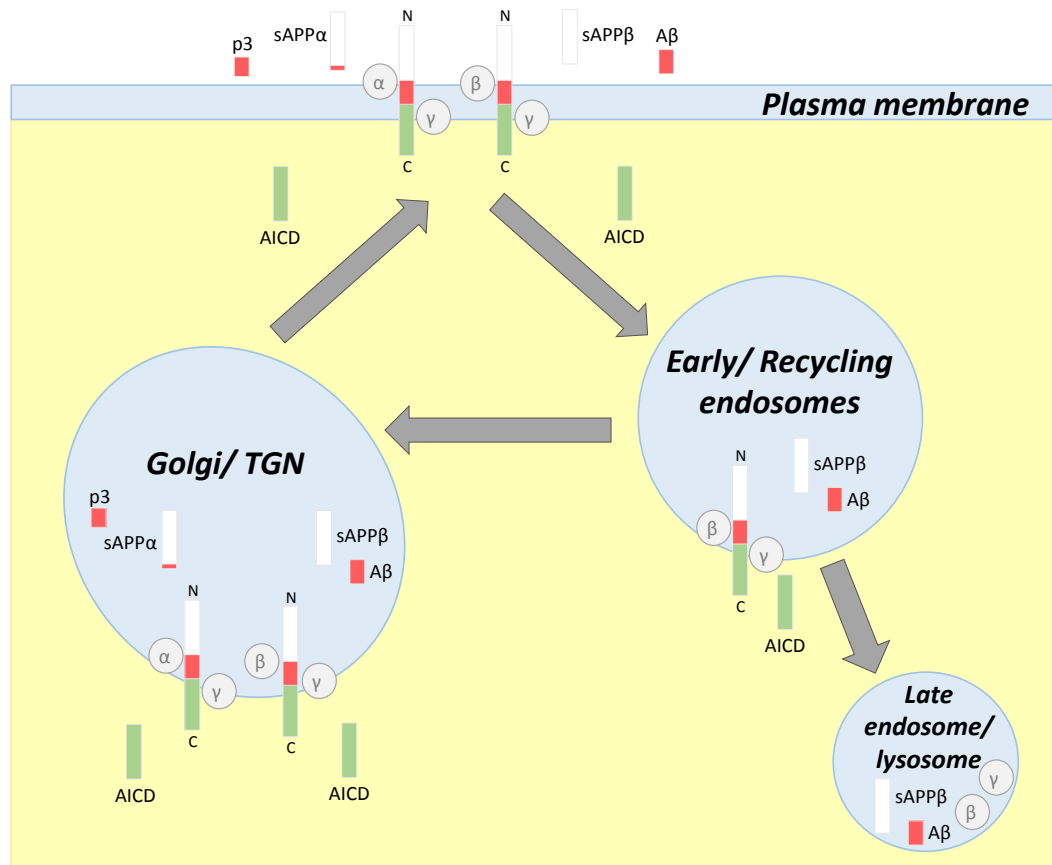


Figure 1.12 Subcellular localisation of amyloidogenic and non-amyloidogenic processing of APP. Full length APP is shown with the N-terminal domain in white, the ‘Aβ’ domain in red, and the C-terminal domain in green. Secretase enzymes are in light grey circles, identified by their prefix. Adapted from (Agostinho et al. 2015).

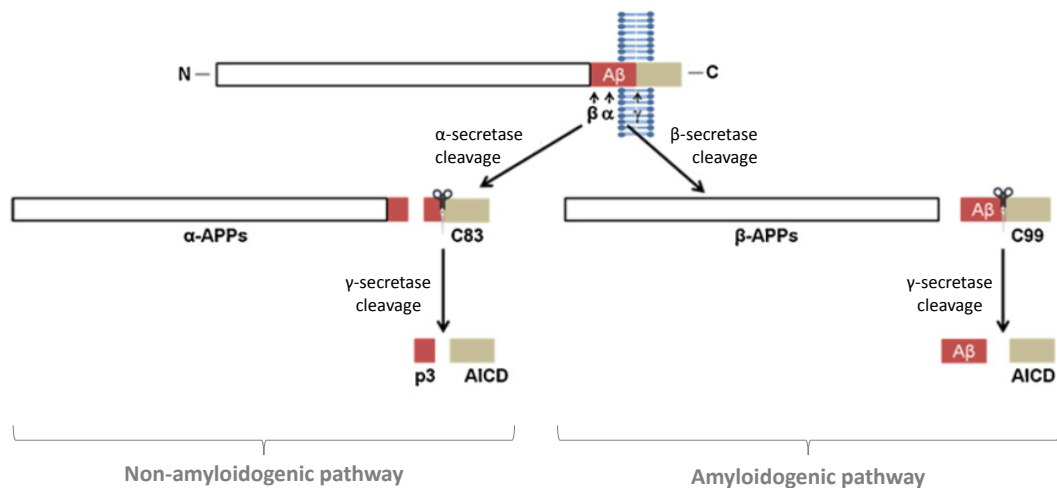


Figure 1.13 Secretase-mediated cleavage of APP, represented schematically. Cleavage by α-secretase produces sAPPα (α-APPs) and C83, whereas cleavage by β-secretase produces sAPPβ (β-APPs) and C99. C83 and C99 can be further metabolised by γ-secretase into p3 and AICD, or Aβ and AICD, respectively. Adapted from (Fodero-Tavoletti et al. 2011).

α -Secretase cleaves APP at the 17th amino acid of the A β sequence. An N-terminal 'sAPP α ' domain is released, leaving α C-terminal fragment (α CTF) in the membrane, which is an 83-amino acid peptide otherwise known as 'C83' (Figure 1.13). Like the amyloidogenic pathway, cleavage of C83 by γ -secretase produces AICD (Zhang et al. 2012). The other peptide created by C83 cleavage, called 'P3' or 'A β 17-42', is not detected in amyloid plaques of AD brains, but is not considered to be entirely benign (Tekirian et al. 1998). P3 is present in diffuse deposits in AD brains, and induces caspase-8-mediated apoptosis in cell lines (Tekirian et al. 1998; Wei et al. 2002).

1.5.3 Function of APP

Since its discovery, full-length APP has been proposed to act as a cell surface receptor, given that APP shares many features with Notch receptors, and putative ligands such as Netrin-1 and F-spondin have been identified. Netrin-1-binding causes APP to complex with FE65, a putative adaptor protein, although the cell signalling pathway itself is yet to be characterised (Zheng & Koo 2011; Lourenço et al. 2009). One suggestion is that the AICD translocates to the nucleus and engages in transactivation of gene expression, in a complex with adaptor FE65 and histone acetyltransferase Tip60 (Cao & Südhof 2001; Kimberly et al. 2001). Doubts have since been cast on the biological relevance of this pathway, particularly given reports that AICD is not essential for the transcriptional activity in question (Zheng & Koo 2011). Nevertheless, several reports have highlighted a specific effect of AICD on neuronal precursor cell proliferation (Lazarov & Demars 2012). For example, expression of AICD in APP knock-out mice appears to reduce adult neurogenesis in an age-dependent manner (Ghosal et al. 2010).

In contrast the sAPP α domain of APP, shed by α -secretase processing, appears to promote neurogenesis (Zheng & Koo 2011). sAPP α promotes the differentiation of a wide variety of cell types in culture, as well as neuronal precursors (Lazarov & Demars 2012). The apparent neurotrophic role of sAPP α may be linked to other observed effects of sAPP α , including differentiation and synaptic plasticity. sAPP α added to differentiating neuronal precursor cells in culture results in enhanced neurite outgrowth (Gakhar-Koppole et al. 2008). Furthermore, a dose-dependent increase in NMDA receptor transmission, during long term potentiation (LTP), was observed in rat hippocampal slices perfused with sAPP α (Taylor et al. 2008). Neurogenic effects also have been reported with full-length APP expression that may be a result of increased sAPP. The function of sAPP β appears to partly overlap with sAPP α . Indeed, one study found that recombinant sAPP β more strongly induces neural differentiation of embryonic stem cells than sAPP α (Freude et al. 2011). On the other hand, another study of

excitotoxic cell stress in hippocampal neurons found that sAPP α had a strong neuroprotective effect whereas sAPP β was about 100-fold less potent (Furukawa et al. 2002; Nhan et al. 2015).

In addition to postsynaptic effects, a presynaptic role for APP has been more recently proposed. Potentially APP may affect neurotransmission as well as synaptogenesis. Both APP and APLP2 have been shown to reside in synaptic vesicles, and although single knock-out mice are healthy, double knock-out mice exhibit severe neuromuscular junction defects (Wang et al. 2005; Fanutza et al. 2015; Del Prete et al. 2014). This suggests that APP and APLP2 have redundant roles in synaptic function. Fanutza et al suggest that APP and ALP2 promote glutamate neurotransmitter release. In double knock-out mouse hippocampal slices, the frequency of reduced miniature excitatory post-synaptic currents is reduced, and the calculated 'probability of release' function reduced. Similar effects could be produced in WT mice using an intracellular-targeted dominant-negative peptide for part of the APP cytoplasmic domain. This region of APP, in the N-terminal part of AICD, appears to specifically interact with synaptic proteins synaptophysin, vesicle-associated membrane protein-2 (Vamp2), and synaptotagmin-2 (Fanutza et al. 2015). A physiological involvement in synaptic vesicle regulation appears likely, but awaits confirmation.

Another emerging physiological function for APP is in the regulation of cellular iron. Iron has long been known to accumulate abnormally in AD brains (Ayton et al. 2013). Furthermore, iron regulates APP expression, via an Iron-Responsive Element (IRE) in the 5' untranslated region of APP mRNA. Reduced intracellular iron levels cause Iron-Regulatory Protein-1 (IRP-1) to bind to the IRE region of APP mRNA, and inhibit translation of APP. Conversely, iron influx into the cell liberates IRP-1 from IREs, and allows more APP protein to be translated (Rogers et al. 2002). APP does not bind iron, and does not have iron redox activity, despite promoting iron efflux from cells when over-expressed (Duce et al. 2010; Ebrahimi et al. 2013; Ebrahimi et al. 2012). Yet full-length APP and sAPP α bind to ferroportin, an exporter of ferrous iron, and appear to stabilise it at the plasma membrane, increasing ferroportin numbers at the surface available for iron export (McCarthy et al. 2014; Wong et al. 2014). This novel role may be one of many independent cell functions of APP that are yet to be discovered.

1.6 Connections between α -syn and APP

1.6.1 Localisation

Close physical proximity would present opportunities for α -syn to directly bind APP or APP-associated proteins. In neurons, APP tends to accumulate in post-synaptic compartments,

and α -syn has a particular affinity for synaptic vesicles (Maroteaux et al. 1988; Hoey et al. 2009). Yet APP is not exclusively post-synaptic and has in several studies been found, with its β -secretase, at synaptic vesicles and the pre-synaptic membrane (Groemer et al. 2011; Del Prete et al. 2014; Laßek et al. 2013). There is therefore an opportunity for α -syn to directly interact with APP in synaptic vesicles, although this has not yet been studied. It is also important to consider potential *indirect* interactions. Synaptic vesicle clustering, docking, and fusion are proposed to be modulated by α -syn, which could affect pre-synaptic APP (Snead & Eliezer 2014). Furthermore, α -syn binds other subcellular membranes with high curvature, and can impede secretory pathway flux when over-expressed in cell models (Snead & Eliezer 2014; Oaks et al. 2013; Thayanidhi et al. 2010). APP processing is tightly regulated through cycling between the plasma membrane, endosomes, and TGN, so changes to secretory pathway flux may disrupt this (Jiang et al. 2014). APP processing is also altered by chronic cell stress (Chami & Checler 2012; Ohno 2014). Pathological changes to α -syn are hypothesised to trigger a variety of cell stress responses due to oxidative stress, mitochondrial dysfunction, calcium dyshomeostasis, or proteasome inhibition (Vekrellis et al. 2011). Despite this, there is limited literature on the subject of α -syn/APP interactions. Evidence for direct or indirect interactions between α -syn and APP is outlined in the following paragraphs.

1.6.2 Human pathology

A connection between α -syn and APP was first suggested by Masliah and colleagues in 2001, who hypothesised “convergent pathogenic effects” of α -syn and β -amyloid (Masliah et al. 2001). Several studies of dementia brains had noted the frequent concurrence of cortical amyloid plaques and Lewy Bodies, with mixed symptoms of AD and PD, described as the ‘Lewy Body Variant’ of AD (Ditter & Mirra 1987; Hansen et al. 1990; Galasko et al. 1994). Since 1996, DLB has been recognised as a distinct disease, and not a variant of AD (McKeith et al. 1996). In fact, DLB is now generally accepted to be on a spectrum with synucleinopathies PDD and PD (Irwin et al. 2013). The dementia symptoms correlate best with levels of cortical Lewy bodies, which appear to spread from the midbrain by cell-to-cell transmission (Irwin et al. 2013). Interestingly, all studied cases of PD involving SNCA mutations and gene multiplications have manifested cortical Lewy bodies and dementia (Poulopoulos et al. 2012). β -Amyloid deposition is common in PDD/DLB, although it is not clear whether this is connected to the α -syn pathology. In patients with severe β -amyloid deposition there appears to be a correlation with cortical Lewy bodies, however it must be noted that both pathologies strongly correlate with age (Irwin et al. 2013; Pletnikova et al. 2005; Irwin et al. 2012; Lashley et al. 2008). One reason to suspect an α -syn/ β -amyloid interaction in DLB, is that DLB amyloid plaques have been reported to contain fragments of α -syn (Yokota et al. 2002; Liu et

al. 2005). Furthermore, co-immunoprecipitation of α -synuclein and A β can be achieved using brain samples from DLB and AD patients. This apparent interaction is absent in ‘non-demented’ control brains (Tsigelny et al. 2008).

1.6.3 In vitro studies

Potential α -syn and A β interactions have been examined *in vitro*. Both proteins have the propensity to form amyloid fibrils, and *in vitro* this can be induced by incubating monomers with ‘seeds’ of β -sheet-rich oligomers (Ono et al. 2012). α -Syn and A β have been shown to participate in ‘cross-seeding’ *in vitro*. This phenomenon, where fibrillisation is stimulated by ‘seeds’ of a completely different protein, owes to the common cross β -sheet structure that all amyloid fibrils contain (Westermarck & Westermarck 2013). In a number of *in vitro* studies, α -syn has been shown to seed A β 40 or A β 42 fibrils, and A β 42 seed α -syn fibril formation (Masliah et al. 2001; Ono et al. 2012; Mandal et al. 2006; Atsmon-Raz & Miller 2015). In addition to fibrils, Tsigelny et al. reported ring-like oligomers resulting from the incubation of A β 42 oligomers with α -syn monomers (Tsigelny et al. 2008). However, cross-seeding *in vitro* has not yet been replicated experimentally *in vivo*. A major attempt to cross-seed amyloid plaques in APP-PS1 transgenic mice using α -syn recombinant ‘pre-formed fibrils’ was unsuccessful. Additionally, APP-PS1 mice were injected intracerebrally with α -syn aggregate-containing brain homogenates from transgenic donor mice. Although seeding of Lewy body-like α -syn inclusions was detected, there was no cross-seeding of amyloid plaques (Bachhuber et al. 2015).

1.6.4 Cell studies

Cell culture studies reveal some evidence of synergistic toxicity between α -syn and A β . Bate et al. used synaptophysin levels to measure synapse damage in mouse primary neurons. Recombinant human α -syn added to the neurons caused synapse damage, which was significantly potentiated by pre-mixing α -syn with A β 42 in a 50:1 or 2:1 ratio. Interestingly, if 1 nM A β 42 or 10 nM α -syn were added to neurons as a pre-treatment to the other peptide, rather than pre-mixing, the synergism was lost (Bate et al. 2010). The implication is that the synergistic toxicity in this model depends on the extracellular stimulus. However it likely obscures the intracellular effects of the peptides. Another group looked more specifically at intracellular interactions, by studying endogenous levels of A β 40 in response to the application of α -syn recombinant aggregates (Majd et al. 2013). A sub-toxic dose of α -syn aggregates increased intracellular and extracellular A β 40 in rat primary neuron cultures. Additionally, reciprocation was apparent when cells were treated with A β 42 aggregates, which increased endogenous α -syn levels (Majd et al. 2013). A similar effect on β -amyloid secretion was

demonstrated in an earlier study using PC12 cells treated with un-aggregated α -syn (Kazmierczak et al. 2008). Stable overexpression of α -syn is a useful tool for studying the chronic effects of α -syn on cells. In SH-SY5Ys, α -syn overexpression has been reported to increase APP expression. β -Amyloid production was not measured (Jesko et al. 2014).

1.6.5 Transgenic mouse models

In transgenic mouse models, attempts have been made to combine high A β production with over-expressed wild-type or mutant α -syn, in order to model DLB with co-morbid AD (Clinton et al. 2010). Two of these models exhibit accelerated neurodegeneration compared with transgenic mice that had a single pathology. Neurodegeneration temporally coincided with increased levels of insoluble A β - or α -synuclein-containing aggregates (Masliah et al. 2001; Clinton et al. 2010). A third mouse model also potentiated synapse loss, but with paradoxical reduction of amyloid pathology. In this study, A30P α -syn transgenic mice were bred with an APP-PS1 mouse model, with high background amyloid burden. The resulting APP-PS1 \times [A30P] α -syn mice had significantly less hippocampal amyloid plaques than APP-PS1 mice, and increased CSF levels of β -amyloid. No changes to β -amyloid secretion were detected in cultured APP-PS1 \times [A30P] α -syn primary neurons. Since synapse loss was nevertheless increased in APP-PS1 \times [A30P] α -syn mice, the reduced amyloid plaque deposition may be detrimental. Oligomers of β -amyloid were not measured, but potentially an inhibition of fibril formation may allow toxic oligomers to accumulate (Bachhuber et al. 2015).

1.7 Future directions

The existing studies of α -syn and A β are both patchy and contradictory. Synergism of their synapse toxicity has been suggested in both cell and rodent models, but the mechanism for this is unknown. Direct interactions between α -syn and A β have been demonstrated *in vitro* and also in material from Lewy Body disease patients (Tsigelny et al. 2008; Ono et al. 2012; Masliah et al. 2001; Mandal et al. 2006). The physical interaction appears *in vitro* to promote oligomer and fibril formation by cross-seeding, but in rodent models may inhibit fibrillisation (Bachhuber et al. 2015). Additionally, an indirect effect of α -syn increasing β -amyloid production and secretion has been proposed. Evidence in favour of this has been obtained from a models of recombinant α -syn protein added to cell cultures (Majd et al. 2013; Kazmierczak et al. 2008). However, a better model to study the chronic intracellular effects of synucleinopathy would be to overexpress α -syn in cells. At present, the effect of α -syn overexpression on β -amyloid production has not been investigated. The field would benefit from more detailed characterisation of the effect of α -syn on β -amyloid production, and the

role of APP expression. Furthermore, the potential cellular mechanism is unexplored, and could one day reveal new therapeutic targets for DLB.

1.8 Aims of the thesis

A clear gap in the literature exists for a comprehensive mechanistic study of β -amyloid production in α -syn overexpression cell models. Broadly, the thesis aims to fit into this niche, and its particular focus on APP secretase-mediated processing is novel in this context. The thesis primarily aims to study in detail how APP secretase-mediated processing is altered by α -syn. The secondary aim is to attempt to find a cell mechanism by which α -syn affects APP processing.

In the literature, neuronal cells treated with recombinant α -syn upregulate β -amyloid secretion (Kazmierczak et al. 2008; Majd et al. 2013). Chapter 3 aims to confirm that overexpression of α -syn in neuronal cells has the same impact on β -amyloid, using a multiplex assay to determine β -amyloid concentration in conditioned media. From this starting point the investigation proceeds in three dimensions: studying the underlying changes to APP amyloidogenic processing, the effect of using different cultured cell models, and the effect of mutating α -syn. α -Syn mutations may have a dominant-negative effect when over-expressed, and could indicate whether changes to APP metabolism result from α -syn function or toxicity.

The two successive chapters aim to make inroads into the mechanism by which α -syn affects APP processing. Chapter 4 narrows the focus onto the secretase enzymes that regulate APP amyloidogenic processing. The chapter aims to ascertain whether α -syn cells exhibit changes to expression and activity of α -, β -, and γ -secretases. The activity of γ -secretase will be studied in-cell with a luciferase reporter. The expression and *in vitro* activity of the β -secretase BACE1 and the α -secretase ADAM10 will furthermore be characterised. Chapter 5 will use the secretase expression/activity phenotype in α -syn cells to identify potential upstream cell signalling targets from the literature. Multiple candidate pathways will be selected from the literature, based on their induction by α -syn, and their ability to affect secretase expression/activity. A primarily pharmacological approach will be used to test the contribution of the candidate pathways to secretase expression/activity, using a single phenotypic readout. Ultimately it is hoped that one or two candidate cell mechanisms will emerge from this initial pass, and be investigated in further detail as potential mediators of the effect of α -syn upon APP processing.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

DMEM high glucose with L-Glutamine, and Ham's F-12 were from Lonza. B-27 AO was from Gibco. V-PLEX Plus A β Peptide Panel 1 (6E10) Kit was from Meso Scale Discovery. FuGene HD and Dual-Reporter Luciferase Assay Kit were from Promega. SensoLyte® 520 ADAM10 and BACE1 Activity Assay Kits were from Eurogentec. Pierce™ Cell Surface Protein Isolation Kit was from Thermo Scientific. TAPI-1, Amyloid Precursor Protein β -Secretase Inhibitor (β SI), and β -Secretase Inhibitor IV (β IV) were from Merck Millipore. DAPT was from Tocris. CM-H₂DCFDA molecular probe was from Life technologies. Dantrolene, PKR inhibitor, salubrinal, and SP600125 were from Santa Cruz. Clastolactacystin- β -lactone, FK-506, and sc-514 were from Enzo Life Sciences.

All other chemicals, including A23187, ammonium chloride, cycloheximide, and tunicamycin, were from Sigma.

2.2 Plasmids

The pFR-Luciferase reporter (pLuc) containing the firefly (*Photinus pyralis*) luciferase gene under the control of a synthetic promoter consisting of five tandem repeats of the yeast GAL4 activation sequence upstream of a minimal TATA box, and pRL-thymidine kinase (pTK) vector containing the sea pansy (*Renilla reniformis*) luciferase gene under the control of the HSV (herpes simplex virus)-TK promoter, were from Promega.

The APP cleavage luciferase reporter (APP-Gal4), a pRC-CMV vector containing a cDNA encoding for human APP695 fused in-frame at its C terminus via a 5 glycine hinge to the yeast transcription factor Gal4 containing both the DNA-binding and activation domains, was kindly provided by Dr. Robert J. Williams (Department of Biology & Biochemistry, University of Bath), as described in (Hoey et al. 2009).

The Notch cleavage luciferase reporter (Notch-Gal4), a pSecTag2 vector containing cDNA encoding for human Notch3 fused in frame at its C terminus to the yeast transcription factor

Gal4 was also kindly provided by Dr. Robert J. Williams (Department of Biology & Biochemistry, University of Bath), as described in (Cox et al. 2014).

Human BACE1 promoter luciferase reporter, a pGL3-Basic vector containing a 4.3 kb fragment of BACE1 promoter from -4372 to -1 of the promoter sequence, was cloned previously (McHugh et al. 2012).

Human ADAM10 promoter luciferase reporter, a pGL3-Basic vector containing a 2.2 kb fragment of ADAM10 promoter from -2179 to -1 of the promoter sequence, was first described in (Prinzen et al. 2005) and was a kind gift from Prof. F. Fahrenholz.

2.3 Cell culture and stable transfections

Transgenic SH-SY5Y, N2A, and HEK293 cell cultures were maintained in media grown in 1:1 DMEM (high glucose with L-Glutamine, Lonza) and Ham's F-12 (Lonza), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.4 mg/ml G418. Growth conditions were maintained at 37°C and 5% CO₂ in a humidified incubator. The full complement of transgenic cell lines used in this thesis are listed in Table 1. Stable transfection of mammalian cells was achieved using FuGene HD transfection reagent (Promega). 25 µl of FuGene HD was incubated with 4.5 µg purified plasmid DNA in 430 µl of DMEM for 12 minutes to form reagent:DNA complexes, which were applied to a T25 flask of cells, plated the day before to reach 50% confluence. 24 hours later, G418 solution was added to a final concentration of 0.8 mg/ml, and the cells maintained in 0.8 mg/ml G418 for 3-4 weeks to select for G418 resistance. Stable expression of the plasmid gene was determined by western blotting.

Cell type	Transgenic cell line		Plasmid	
	Name	Source	Name	Reference
SH-SY5Y	Empty vector/ pcDNA	Dr Jennifer McDowall	pcDNA3.1(+)	Invitrogen, UK
	WT (v1)	Dr Jennifer McDowall	pcDNA3.1(+)- α -syn	X. Wang et. al., 2010
	WT (v3)	Miss Hazel Roberts	pcDNA3.1(+)- α -syn	X. Wang et. al., 2010
	WT (v4)	Miss Hazel Roberts	pcDNA3.1(+)- α -syn	X. Wang et. al., 2010
	Δ 2-9	Prof. David Brown	pcDNA3.1(+)- α -syn Δ 2-9	X. Wang et. al., 2010
	Δ NAC	Miss Hazel Roberts	pcDNA3.1(+)- α -syn Δ 71-82	Previously made by site-directed mutagenesis ^A
	A30P	Prof. David Brown	pcDNA3.1(+)- α -syn A30P	X. Wang et. al., 2010
	E46K	Miss Hazel Roberts	pcDNA3.1(+)- α -syn E46K	X. Wang et. al., 2010
	A53T	Prof. David Brown	pcDNA3.1(+)- α -syn A53T	X. Wang et. al., 2010
	S129A	Dr Jennifer McDowall	pcDNA3.1(+)- α -syn S129A	Previously made by site-directed mutagenesis ^B
	S129D	Dr Jennifer McDowall	pcDNA3.1(+)- α -syn S129D	Previously made by site-directed mutagenesis ^C
	β -syn	Dr Jennifer McDowall	pcDNA3.1(+)- β -syn	Wright, McHugh, Pan, Cunningham, & Brown, 2013
	Steap3	Miss Ye Ding	pcDNA3.1(+)-Steap3	Previously cloned ^D
	pCI	Miss Hazel Roberts	pCI-neo	Prof. Christopher Miller, KCL ^E
	APP	Miss Hazel Roberts	pCI-neo-APP ₆₉₅	Prof. Christopher Miller, KCL ^E
N2A	Empty vector/ pcDNA	Miss Hazel Roberts	pcDNA3.1(+)	Invitrogen, UK
	WT	Miss Hazel Roberts	pcDNA3.1(+)- α -syn	X. Wang et. al., 2010
	Δ 2-9	Miss Hazel Roberts	pcDNA3.1(+)- α -syn Δ 2-9	X. Wang et. al., 2010
	E46K	Miss Hazel Roberts	pcDNA3.1(+)- α -syn E46K	X. Wang et. al., 2010
HEK293	Empty vector/ pcDNA	Miss Hazel Roberts	pcDNA3.1(+)	Invitrogen, UK
	WT	Miss Hazel Roberts	pcDNA3.1(+)- α -syn	X. Wang et. al., 2010
	Δ 2-9	Miss Hazel Roberts	pcDNA3.1(+)- α -syn Δ 2-9	X. Wang et. al., 2010
	E46K	Miss Hazel Roberts	pcDNA3.1(+)- α -syn E46K	X. Wang et. al., 2010

Table 1 Details of all transgenic cell lines used. ^A Primers: 5'-CAAATGTTGGAG-GAGCAGTGGAGGGAGCAGGGAGCA-3' and 5'-TGCTCCCTGCTCCCTCCACTGCT-CCTCCAACATTTC-3'. ^B Primers: 5'-GAAATGCCTGCTGAGGAAGGG-3' and 5'-CC-CTTCCTCAGCAGGCATTTC-3'. ^C Primers: 5'-GAAATGCCTGATGAGGAAGGG-3' and 5'-CCCTTCCTCATCAGGCATTTC-3'. ^D RefSeq accession number NG_042823.1. ^E Detailed in (Lau et al. 2000)

2.4 Western blotting

For extraction of the total complement of cellular proteins, confluent T25 flasks or 6-well plates of cells were lysed in 180 μ l or 100 μ l of cold PBS with 0.5% Igepal CA-630 and 'complete' protease inhibitor cocktail (Roche) respectively. Lysates were scraped into 0.5 ml centrifuge tubes on ice, sonicated 3 x 3 seconds, and centrifuged 10 000 xg for 3 minutes. Supernatants were removed and the pellets discarded. A Bio-Rad protein assay was used with

CHAPTER 2: MATERIALS AND METHODS

1:20 dilutions of the supernatants to determine their protein concentration, according to the manufacturer's instructions (Bradford 1976). Supernatant protein concentrations were normalized, and were boiled for 5 minutes with 1 x Laemmli SDS-PAGE buffer. Samples were loaded into 12% acrylamide gels for Tris SDS-PAGE, run at 250V/35 mA for 45 minutes. The separated proteins were transferred to a PVDF membrane by a semi-dry transfer apparatus, run at 25V/100 mA for 1.3 hours. Membranes were blocked in 5% w/v non-fat milk powder in TBS-T for 30 minutes, incubated with primary antibody for 1-2 hours, and washed 3 x 5 minutes in TBS-T. Primary antibodies are listed in Table 2. Membranes were blocked again and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour. A further 3 x 10 minute washes were performed, and the membranes developed with Luminata Crescendo or Luminata Forte ECL substrate (Thermo Scientific), and imaged with either X-ray film (Amersham) or a Fusion SL CCD imaging system (Vilber Lourmat). Band intensities were quantified using Fusion-Capt Advance software (Vilber Lourmat). Raw data was normalised by division with the mean OD of the experiment for that antibody, and then the optical density (OD) of the 'test' antibody divided by the OD of the 'housekeeping' antibody (usually α -tubulin). Each western used 2 technical replicates.

Protein target	Species raised	ID	Source	Dilution
α -synuclein	Rabbit	MJFR1	Abcam	1:5000
α -synuclein	Mouse	610787	BD Biosciences	1:2000
α -tubulin	Mouse	T5168	Sigma	1:10 000
β -synuclein	Mouse	ab167607	Abcam	1:500
ADAM10	Rabbit	ab10926	Millipore	1:2000
APP C-terminus	Rabbit	Y188	Abcam	1:2000
BACE1	Rabbit	D10E5	Cell signalling technology	1:1500
eIF2 α	Rabbit	9722	Cell signalling technology	1:2000
Phospho-eIF2 α	Rabbit	9721	Cell signalling technology	1:1000
Steap3	Rabbit	72513	Abcam	1:1000

Table 2 Details of antibodies used for western blotting.

2.5 Luciferase reporter assays

Cells were plated to 40% confluence the day before transfection in 24-well plates, and transfected with plasmid DNA using 1.5 µl of FuGene HD (Promega) per well. Plasmids are listed in Table 3. The APP-Gal4 luciferase based reporter assay was as previously described (Hoey et al. 2009), and used 50 ng APP-Gal4 plasmid co-transfected with 50 ng pLuc per well. Figure 2.1 illustrates how the assay works. The Notch-Gal4 luciferase based reporter assay was as previously described (Cox et al. 2014), and follows similar principles, except using 100 ng Notch-Gal4 plasmid co-transfected with 50 ng pLuc per well. For the BACE1 (McHugh et al. 2012) and ADAM10 promoter reporter assays (Prinzen et al. 2005), 200 ng plasmid was used per well. All reporter plasmids were additionally co-transfected with 50 ng pTk, as an internal transfection control. Any chemical or pharmacological treatments were diluted in serum- and antioxidant-free DMEM supplemented with ‘B-27 AO’ (Gibco), and applied to the cells 6 hours post-transfection. Luciferase activity was measured 20-22 hours post-transfection using the Promega Dual-Luciferase Report Kit as per the manufacturer’s instructions. Assays were carried out using a FLUOstar Omega plate spectrophotometer (BMG Labtech). Raw data was normalised by division with the mean firefly or *Renilla* luminescence for the experiment. Relative Luciferase Units (RLU) for each well were then calculated by division of the firefly signal by the *Renilla* signal. Each experiment used 3-5 biological replicates.

Plasmid	Quantity plasmid required for assay/ ng			
	APP cleavage	Notch cleavage	ADAM10 promoter activity	BACE1 promoter activity
pTK	50	50	50	50
pLuc	50	50	-	-
APP-Gal4	50	-	-	-
Notch-Gal4	-	100	-	-
ADAM10 promoter	-	-	200	-
BACE1 promoter	-	-	-	200

Table 3 Details of plasmid combinations used for luciferase reporter assays.

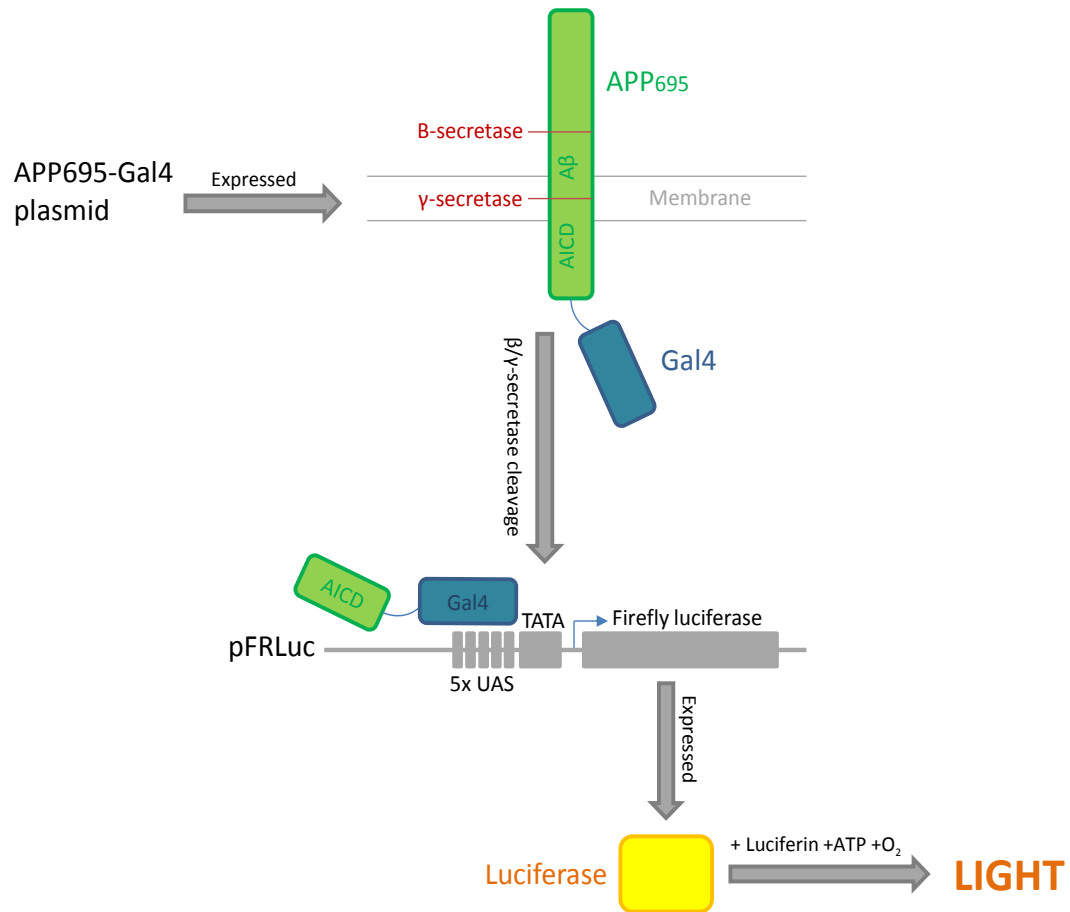


Figure 2.1 Illustration of the APP-Gal4 luciferase reporter assay for β -/ γ -cleavage of APP. Plasmids pFRLuc and APP695-Gal4 were co-transfected into SH-SY5Y cells. APP695-GAL4 fusion protein is cleaved by endogenous secretases, liberating AICD-GAL4 peptides. AICD-GAL4 activates transcription and expression of the inducible firefly luciferase gene of the pFRLuc plasmid. Relative levels of luciferase enzyme were measured by luminometer after addition of luciferin substrate, which triggers a chemiluminescent reaction.

2.6 SDS-PAGE for APP C-terminal fragments

The overall workflow for this is described in Figure 2.2. Cells were plated to 50% confluence in 6 well plates, and after 24 hours were treated with 2 μ M DAPT in B-27 supplemented DMEM for 16 hours. 80 μ L of PBS with 0.5% Igepal CA-630 and ‘complete’ protease inhibitor cocktail (Roche) was used to lyse the cells. Lysates were scraped into 0.5 ml centrifuge tubes on ice, sonicated 3 x 3 seconds, and centrifuged 10 000 xg for 3 minutes. Supernatants were removed and the pellets discarded. A Bio-Rad protein assay was used with 1:20 dilutions of the supernatants to determine their protein concentration, according to the manufacturer’s instructions. Supernatant protein concentrations were normalized, and they

were boiled for 5 minutes with Laemmli buffer. Samples were loaded onto a 16% acrylamide gel (16% Acrylamide (v/v), 990 mM Tris-HCl/SDS pH 8.45, 10% Glycerol (v/v), 0.025% APS (w/v), 0.05% TEMED (v/v)), which was placed into an electrophoresis tank with an anode buffer of 200 mM Tris-HCl pH 8.9, and a cathode buffer of 100 mM Tris, 100 mM Tricine, and 1% SDS (w/v). The 16% gel was electrophoresed at 100 V, 4 °C, for several hours. Western blotting was then performed as described previously.

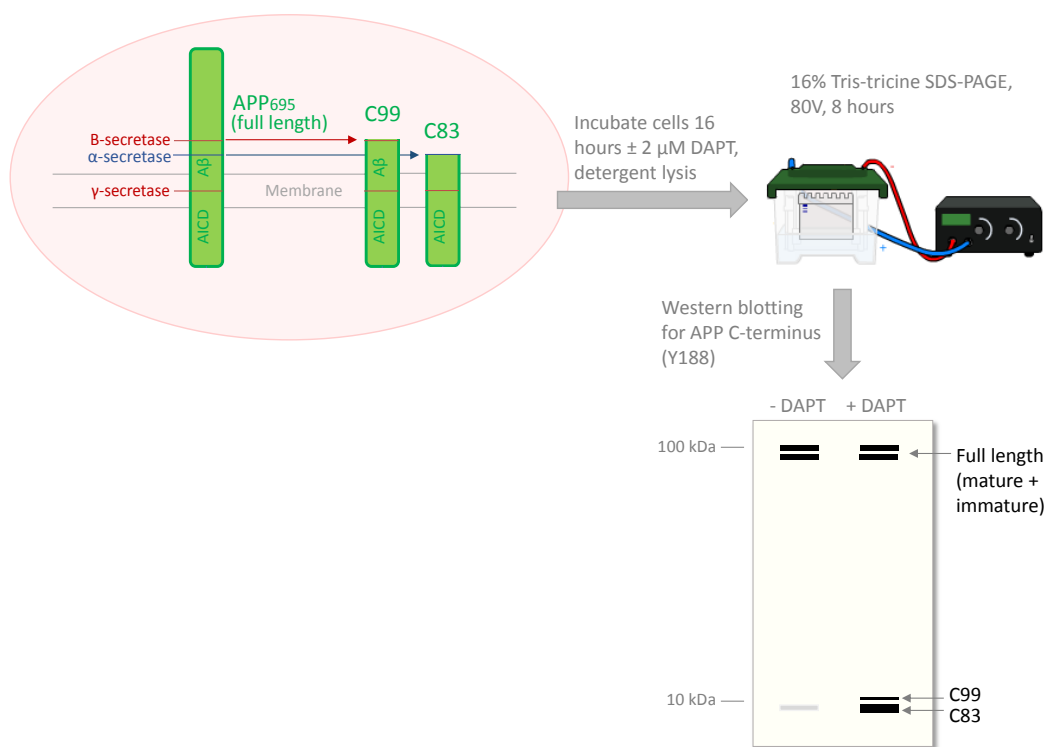


Figure 2.2 Illustration of western blotting for APP C-terminal fragments. Incubation with γ-secretase inhibitor (DAPT) allows the β-cleavage APP CTFs (C99) and α-cleavage APP CTFs (C83) to accumulate to a measurable level before cell lysis. Cell proteins are separated on a 16% SDS-PAGE gel and immunoblotted for the C-terminus of APP (Y188, Abcam).

2.7 Meso Scale Discovery multiplex assay for secreted Aβ₄₀ and Aβ₄₂

Cells were seeded onto 24-well plates at a range of densities (30-60% confluence) in full media, and after 24 hours changed to 800 μl serum-free B-27-supplemented DMEM, with compounds where indicated. Conditioned media was collected after 72 hours and immediately assayed without further manipulation, using the V-PLEX Plus Aβ Peptide Panel 1 (6E10) Kit from Meso Scale Discovery, according to the manufacturer's instructions (Figure 2.3). The plate was read with a Sector Imager 6000 (Meso Scale Discovery). Peptide concentrations

were calculated by Meso Scale Discovery Workbench software, with reference to a standard curve. Data was normalised by division with the mean peptide concentration of the experiment.

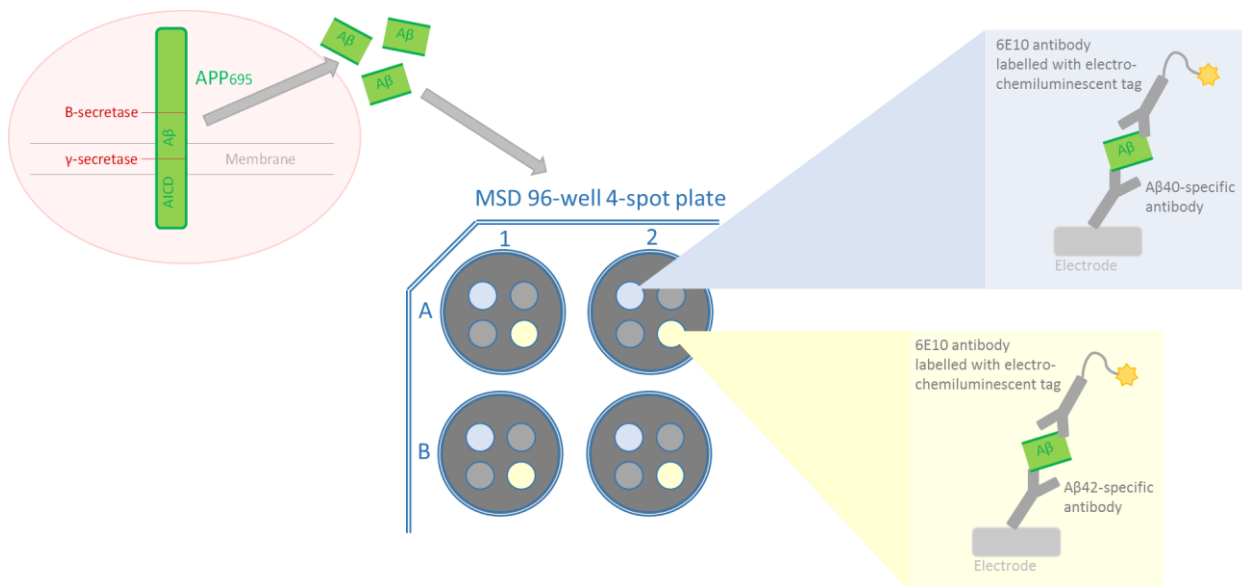


Figure 2.3 Illustration of the Meso Scale Discovery multiplex assay for Aβ40 and Aβ42 peptides. An aliquot of conditioned media from 72-hours of cell culture is applied to the MSD 96-well 4-spot plate. Each well contains spots of immobilized capture antibody specific for Aβ40 and Aβ42 individually, which bind peptides from the media. After washing the plate, the ‘sulfo-tag’ 6E10 detection antibody is applied, which binds any affixed Aβ peptide and forms a sandwich. Electrification of the plate triggers an electro-chemiluminescent reaction in the ‘sulfo-tag’ of the 6E10. Adapted from the assay protocol (Meso Scale Discovery).

2.8 Immunofluorescent staining and confocal microscopy for α-synuclein

Cells were seeded at a density of approximately 1×10^6 cells /ml onto poly-D-lysine-coated coverslips in a 24-well plate. After 24 hours, the coverslips were washed with PBS/CM (10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl , 2.7 mM KCl , 0.9 mM CaCl_2 , 0.33 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), then fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After two washes with PBS/CM, permeabilisation was performed in 0.1% Triton X-100 (in PBS/CM) for 10 minutes at room temperature. Coverslips were blocked for 30 minutes in 10% FBS in PBS/CM, and incubated overnight at 4 °C with the primary antibody MJFR1 (1:100, Abcam, #ab138501) for α-syn. Primary antibody was diluted in wash buffer: 2% FBS + 0.05%

Triton X-100 in PBS/CM. Coverslips were then washed three times with wash buffer and three times with PBS/CM. The secondary antibody anti-rabbit IgG-AlexaFluor®568 (1:10,000, Abcam #ab175471) was applied for 1 hour at room temperature. Coverslips were washed three times in PBS/CM, and mounted on slides with Mowiol mounting media and a DAPI counterstain (600 nM). Slides were stored in a light protective box overnight and examined on a Zeiss LSM510 Meta at the relevant wavelengths (AlexaFluor®568 Ex/Em= 578/603 nm, DAPI Ex/Em= 364/454 nm).

2.9 Fluorometric BACE1 and ADAM10 activity assays

The β -secretase activity of BACE1 from cell homogenates was measured using the 'SensoLyte® 520 β -Secretase Assay Kit' (Eurogentec), and the secretase activity of ADAM10 measured using the 'Sensolyte® 520 ADAM10 activity assay kit' (Eurogentec). Cell homogenates were obtained by addition of 60 μ l/well of the ADAM10 kit buffer to confluent 6-well plates. Cell material was scraped into centrifuge tubes, homogenised by pipetting, and incubated on ice for 10 minutes. Lysates were clarified by centrifuging 10 000 xg for 5 minutes, and total protein concentrations quantified by Bradford assay. 150 μ g of total protein was prepared in 150 μ l of kit assay buffer, and 50 μ l loaded onto a black optical 96-well plate. The BACE1 or ADAM10 fluorometric substrate was prepared and added, according to the kit instructions, and fluorescent readings (Ex/Em=490/520 nm) taken in a FLUOstar Omega plate spectrophotometer (BMG Labtech) at every 5-10 minutes for one hour. Raw fluorescence at 30 minutes was averaged for duplicate wells, and normalised to the mean for the experiment.

2.10 Cell surface biotinylation assay for plasma membrane localization of ADAM10

The Pierce™ Cell Surface Protein Isolation Kit (Thermo Scientific #89881) was used to biotinylate intact cells, lyse, and purify biotinylated proteins. Confluent 6-well plates of cells were washed twice with cold PBS/CM (refer to section 2.8), and incubated with 0.5 mg/ml sulfo-NHS-SS-biotin on ice for 30 minutes. Free biotin was quenched with 50 μ l of Quenching Solution (Thermo Scientific), and cells scraped into centrifuge tubes and pelleted by centrifugation at 500 xg for 3 minutes. The cell pellet was washed once with TBS, then resuspended in 180 μ l cold lysis buffer. Lysates were sonicated on a low power with 5 brief pulses, and incubated 30 minutes on ice. Large debris was pelleted and discarded, and the protein concentration of the supernatant quantified by Bradford assay. Samples were adjusted to have equal concentrations, and a 40 μ l aliquot removed to serve as an input control for SDS-PAGE, boiled with Laemmli buffer. The rest of the supernatant was incubated with

Neutravidin beads (Thermo Scientific), overnight at 4 °C with rotation. Beads were collected by centrifugation at 1000 xg for 3 minutes and washed three times with ice-cold Wash Buffer (Thermo Scientific). The biotin-labelled proteins were eluted from the beads by boiling with 80 µl of Laemmli buffer (containing 50 mM DTT), at 100 °C for 15 minutes. Samples were run on an SDS-PAGE gel, and western blotting for ADAM10 performed, as described above.

2.11 Fluorometric assay for reactive oxygen species using CM-H₂DCFDA

Cells were seeded at a density of approximately 1×10^6 cells/ml onto a poly-D-lysine-coated 48-well plate. After 48 hours, cells were incubated with 10 µM CM-H₂DCFDA probe in HEPES-buffered media (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM NaHCO₃, 1.2 mM Na₂HPO₄, 1.2 mM CaCl₂, 5.5 mM glucose) for 20 minutes. The probe was removed and 300 µl of HEPES-buffered media added. Fluorescence intensity (Ex/Em= 488/534 nm) was measured every 5-10 minutes for 60 minutes. The linear rate equation was determined from a kinetics plot using MS Excel, and the rate at 60 minutes calculated. Individual rates were normalised to the mean rate of the experiment.

2.12 Statistical analysis

Student's t tests were two-tailed and performed using MS Excel, with an assumption of equal variance. Pairwise t-tests with Holm adjustment, and one-way ANOVA with post-hoc Tukey HSD, were performed using 'R Studio'. Normal distribution was assumed, and the homogeneity of variances were assessed using a Bartlett's test in 'R Studio'. Differences between treatments were defined as statistically significant when $p < 0.05$.

CHAPTER 3: EFFECT OF α -SYN ON THE AMYLOIDOGENIC PROCESSING OF APP

3.1 Introduction

3.1.1 *Effect of α -syn on APP and β -amyloid*

A number of studies have proposed potential interactions of α -syn with β -amyloid fragments of APP. These have largely focussed on pathology: synergism of toxicity when the recombinant proteins are added to cells (Bate et al. 2010), and synergism of the aggregation of toxic oligomers and amyloid fibrils *in vitro* (Ono et al. 2012; Tsigelny et al. 2008; Atsmon-Raz & Miller 2015). This focus on aggregation could be misguided given the recent evidence that α -syn overexpression appears to reduce the formation of amyloid plaques in mice. The same study found no changes to β -amyloid secretion from primary neurons with over-expressed A30P α -syn (Bachhuber et al. 2015). However, the result was obtained using *APPPS1* mice that already have abnormally high β -amyloid, which would easily mask any subtler effects of α -syn.

Very few studies address the potential interactions between α -syn and APP in cells. There is some evidence in the literature that α -syn affects β -amyloid generation in cells. Enhanced β -amyloid production was observed when cell cultures were treated with toxic levels of unaggregated α -syn (10 μ M), or sub-toxic levels of α -syn aggregates (1 μ M) (Kazmierczak et al. 2008; Majd et al. 2013). Stable overexpression of α -syn in SH-SY5Ys was reported to increase APP expression. However it was not established whether this affected β -amyloid production of the cells (Jesko et al. 2014). This gap in the literature will be addressed within the current chapter, which will measure β -amyloid production in an α -syn cell overexpression model. Amyloidogenic processing of APP will additionally be probed, this has not previously been studied in α -syn cell models.

3.1.2 *The N-terminal domain of α -syn in function*

The chapter will also investigate a number of α -syn mutations, and how they influence the generation of β -amyloid. Truncations of the α -syn N-terminal domain will be investigated.

The N-terminal domain spans the first 95 amino acids of the 140 amino acid sequence of α -syn. It is distinguished from the C-terminal domain by its tendency to form secondary structure. In a membrane-associated state, the N-terminal domain interconverts between a single extended α -helix, and a pair of amphipathic α -helices separated by a flexible linker: 'Helix 1' (3-37 aa) and 'Helix 2' (45-92 aa) (Ulmer et al. 2005).

Deletion of either helix sequence has been shown to reduce the binding of α -syn to artificial membranes. Furthermore this appears to translate into reduced synaptic targeting of α -syn in primary neurons (Burré et al. 2012). Some studies have found that just a small section of the extreme N-terminus, encompassing the first 8 to 11 amino acids, is vital for membrane binding. Evidence from *in vitro*, isolated mitochondria, and yeast experiments indicate a loss of membrane binding with a Δ 2-8 or Δ 2-11 deletion (Robotta et al. 2012; Vamvaca et al. 2009; Burré et al. 2012). However, little effect of Δ 2-11 on membrane binding is seen in neuronal cell lines (Vamvaca et al. 2011; Wang et al. 2010). Rather than reducing α -syn membrane association, it is likely that Δ 2-8/ Δ 2-11 affects the subcellular localisation of α -syn. In primary neurons the synaptic targeting of α -syn is significantly reduced by Δ 2-8 (Burré et al. 2012). The function of α -syn as a SNARE complex chaperone is proposed to require the N-terminal domain for membrane-binding, and the C-terminal domain to interact with synaptobrevin-2 (Burré et al. 2010). Deletion of either, or both, of these sequences strongly disturbs the formation of SNAP25-reactive SNARE complexes, when lentivirally-expressed in primary neurons. Counterintuitively, although the Δ 2-8 mutation reduces α -syn synaptic targeting, it does not significantly affect SNARE complex formation (Burré et al. 2012).

In addition to interacting with membranes, the N-terminal domain of α -syn may interact with other proteins. Calmodulin has been shown to interact with the first 20 amino acids of the α -syn N-terminus (Gruschus et al. 2013). The calcium-bound form of calmodulin appears to compete with membranes for binding to α -syn, and their shared involvement in regulating exocytosis suggests that the interaction may have a biological role (Gruschus et al. 2013; Snead & Eliezer 2014; Lee et al. 2002).

3.1.3 The N-terminal domain of α -syn in toxicity

As well as being important for the putative physiological function of α -syn, the N-terminal domain is implicated in disease. The central section of α -syn, residues 61-95, contains the 'non-amyloid component' (NAC) sequence. Although normally within the helical N-terminal region, in disease NAC forms the β -sheets at the core of toxic oligomers and amyloid fibrils of α -syn (Li et al. 2002). Removal of the NAC from α -syn makes it incapable of aggregating into toxic oligomers and is neuroprotective, as shown in *Drosophila* models (Periquet et al.

2007). In this chapter, NAC-truncated α -syn mutant will be utilised, with the aim of preventing oligomer-mediated toxicity.

This chapter will also feature toxicity-inducing A30P, E46K, and A53T point mutations, discovered in familial PD or DLB (Krüger et al. 1998; Zarranz et al. 2004; Polymeropoulos et al. 1997). The biophysical properties of these mutant proteins may be important for understanding the toxicity of α -syn. The point mutations endow different changes to the membrane affinity of α -syn: E46K enhances the membrane affinity of α -syn, A53T has no effect, and A30P reduces it (Bodner et al. 2010). *In vitro* fibrillisation of the disease mutations is also different: A53T and E46K fibrillise faster than wildtype protein whereas A30P fibrillises slower. Yet a common feature is toxic oligomer formation. All three mutants recruit monomers from solution to form soluble oligomeric species at a faster rate than the wildtype (Conway et al. 2000; Choi et al. 2004).

A biophysical explanation for the behaviour of α -syn disease-associated point mutants has been proposed to involve destabilisation of the N-terminal domain. When membrane-bound, the N-terminal α -helix may be unusual short, only 25 residues. This would expose an unstructured NAC region, and potentially encourage β -sheet interactions between adjacent α -syn molecules (Bodner et al. 2010). Recently another explanation has been proposed, which is that the mutations reduce the safe sequestration of cytosolic α -syn. In the neuronal cytosol α -syn exists predominantly as a non-aggregating tetramer (Bartels et al. 2012; Dettmer et al. 2013). *In silico* modelling of tetramers appears to show that disease-associated point mutations would interfere with tetramer assembly and promote monomeric α -syn in solution (Kara et al. 2013). Hypothetically, the increase in free disordered monomers should encourage β -sheet rich α -syn oligomers to form. The predicted effect of reduced tetramer: monomer ratios was eventually verified in a variety of disease mutant-expressing cell models, including A53T iPSCs, using intact-cell crosslinking and fluorescent protein complementation methods (Dettmer et al. 2015).

Aggregation of α -syn into toxic oligomers is also promoted by copper, which co-ordinates with the extreme N-terminus of α -syn in both the membrane and cytosol (Wright et al. 2009; Wang et al. 2010; Dudzik et al. 2013). Removal of the 2-9 residues creates an aggregation-resistant α -syn protein, that protects against copper-induced toxicity in neuroblastoma cells (Wang et al. 2010).

3.1.4 Role of C-terminal phosphorylation of α -syn in disease

The C-terminus of α -syn is unstructured and negatively charged. When α -syn is membrane-associated the C-terminal domain may be a site for regulatory protein-protein interactions. However in cytosolic form the C-terminal domain may be auto-inhibitory, forming long-range interactions that shield the NAC region from solution (Bertoncini et al. 2005). Post-translational modifications of the C-terminal domain can have a profound effect on α -syn. Calpain-1 cleavage of the C-terminus generates pro-aggregatory 1-120 α -syn, which is a normal cellular process that appears upregulated in synucleinopathy brains (Li et al. 2002; Li et al. 2005). Another modification of the C-terminus linked with disease is phosphorylation of serine-129. S129 phosphorylation is an uncommon and transient modification in normal cells, but was discovered to occur on around 90% of the α -syn protein in urea-insoluble extracts of DLB brains (Fujiwara et al. 2002). Although long linked with pathology the biological role of this modification is unclear, but it appears to mediate autophagic clearance of α -syn (Oueslati et al. 2013), and may be involved in α -syn interactions with vesicle trafficking proteins (McFarland et al. 2008).

This chapter will include S129A and S129D α -syn in the investigation of APP processing. Point mutations S129D and S129A have been used in cell models to artificially mimic or block the phosphorylation site respectively (Chen & Feany 2005). S129 mutations do not affect α -syn aggregation itself (Lázaro et al. 2014), but S129A fails to induce the autophagic clearance of insoluble α -syn aggregates in yeast (Tenreiro et al. 2014). Controversy still exists over whether S129A is neuroprotective (Chen & Feany 2005; Kragh et al. 2009; Febbraro et al. 2013), or promotes neurodegeneration (Gorbatyuk et al. 2008; Kuwahara et al. 2012), or has no effect on toxicity (Sato et al. 2011; McFarland et al. 2009). A recent paper suggests that S129 phosphorylation does not alter the overall dopaminergic neuron loss caused by synucleinopathy in rodent models, but does modulate the rate of disease progression. S129A slowed the loss of striatal dopaminergic terminals, and S129D accelerated neurodegeneration relative to WT α -syn (Febbraro et al. 2013).

3.2 Aims

The aim of this chapter is to characterise the impact of α -syn expression on APP amyloidogenic processing, in stable transgenic cell lines. Initially, levels of secreted β -amyloid will be determined in α -syn overexpressing SH-SY5Ys. The amyloidogenic processing of APP will then be measured with two complementary techniques: (A) Indirect quantitation of amyloidogenic processing, performed with a luciferase reporter for endogenous β - and γ -secretase activity; (B) A direct, semi-quantitative method, using western blotting to detect

cell levels of C99, a peptide precursor to β -amyloid. Next, α -syn-induced changes to APP processing will be studied in other cell lines: the mouse neuronal cell line ‘N2A’, and human fibroblast-like cell line ‘HEK293’. Finally, the role of α -syn primary structure in mediating effects to APP processing will be investigated, using a dominant-negative mutant approach. Mutants include α -syn lacking sections of the N terminal domain, $\Delta 2-9$ and $\Delta 71-82$ (henceforth known as ‘ Δ NAC’). Disease-associated point mutations A30P, E46K, and A53T, will also be included. Additionally, the role of phosphorylation at S129 will be examined with S129A and S129D α -syn SH-SY5Ys, to block or mimic serine phosphorylation respectively. All mutants are illustrated in Figure 3.1.

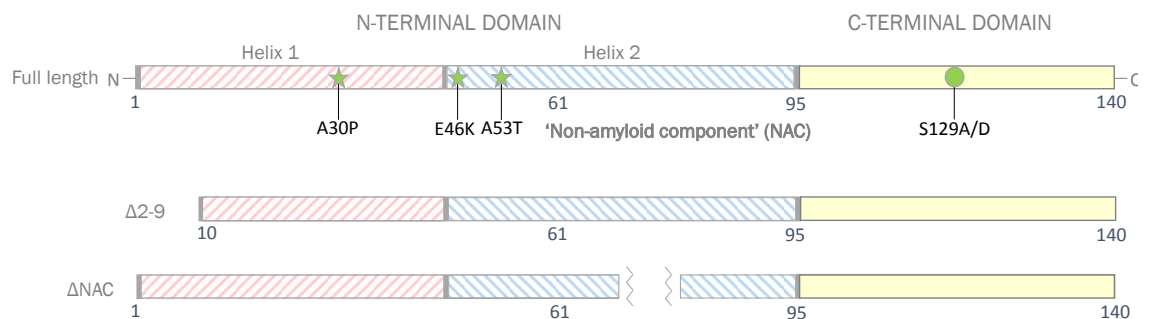


Figure 3.1 Schematic diagram of the mutations of α -syn over-expressed in SH-SY5Ys.

The N-terminal domain is residues 1-95 and consists of a maximum of two α -helices in the membrane-bound state, as indicated with diagonal stripes. The ‘non-amyloid component’ region is residues 61-95. Disease-associated point mutations are indicated by stars, and the phosphorylation mutants by an oval. Truncation mutants are displayed beneath. Adapted from Burré et al (Burré et al. 2012).

3.3 Results

3.3.1 *Stable overexpression of α -syn was achieved in three independent SH-SY5Y lines*

SH-SY5Ys were transfected with pcDNA 3.1 (+)- α -syn (Wang et al. 2010) and selected for G418-resistance, to generate a polyclonal population of stably-transfected cells. Polyclonal selection avoids any phenotypic effects specific to the site of recombination, however the resulting population is not 100% transgenic. A few SH-SY5Y cells are able to develop resistance to G418 in the absence of plasmid, and this non-transgenic subset of cells can vary in proportion over time and between cell lines. To control for the inherent instability of a single polyclonal line, three independent WT α -syn SH-SY5Y lines were produced: 'WT (v1)', 'WT (v3)', and 'WT (v4)'. For β -amyloid measurement and some minor experiments only WT (v1) was used, but key experiments were performed using all three WT lines to ensure reproducibility. Robust 6- to 9-fold over-expression of α -syn was achieved in the three WT lines (Figure 3.2). Yet expression of α -syn is significantly lower in the WT (v1) line compared with the other two, when measured over a two-week period. In a polyclonal line, the level of protein overexpression may be lessened by proliferation of non-transgenic cells. Western blotting does not provide information about the proportion of transgenic cells in each line, but this was estimated by immunofluorescent staining for α -syn. Cells stained positive for α -syn were counted from 20x confocal microscope images, and compared with the number of DAPI⁺ nuclei (Figure 3.3). The population of non-transgenic cells is negligible in the 'WT (v3)' and 'WT (v4)' lines, but appeared to be more than a third of cells in the 'WT (v1)' line. The estimate is a snap-shot from a single point in time, but may account for the lower α -syn expression on average in the 'WT (v1)' line. A fourth line named 'WT (v2)' was discarded, due to more than 80% of the cells appearing to be non-transgenic.

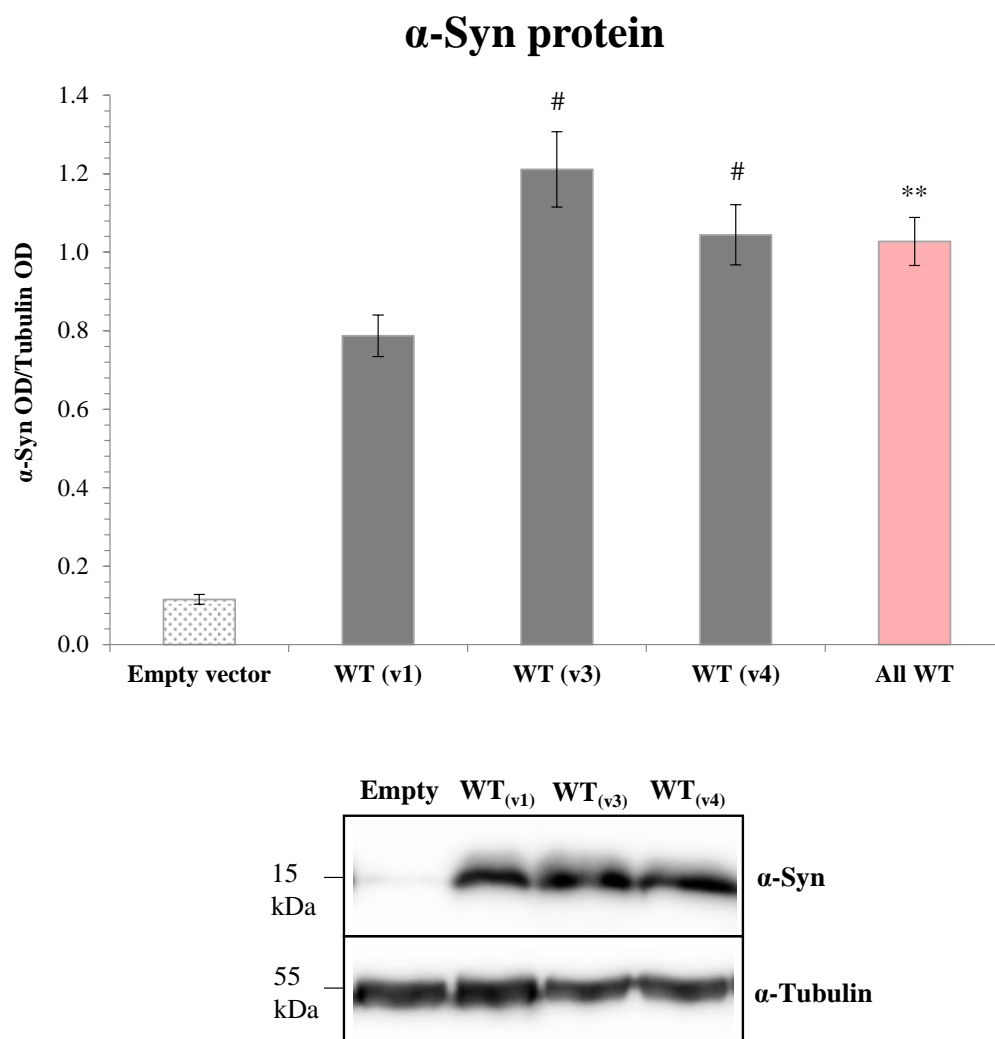


Figure 3.2 Levels of over-expressed α -syn in three lines of WT α -syn SH-SY5Ys. Whole cell lysates were tested for α -syn and α -tubulin by western blotting. Mean α -syn OD: α -tubulin OD for 5 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector, # $p < 0.05$ relative to WT (v1) calculated by pairwise t-tests with a Holm adjustment.

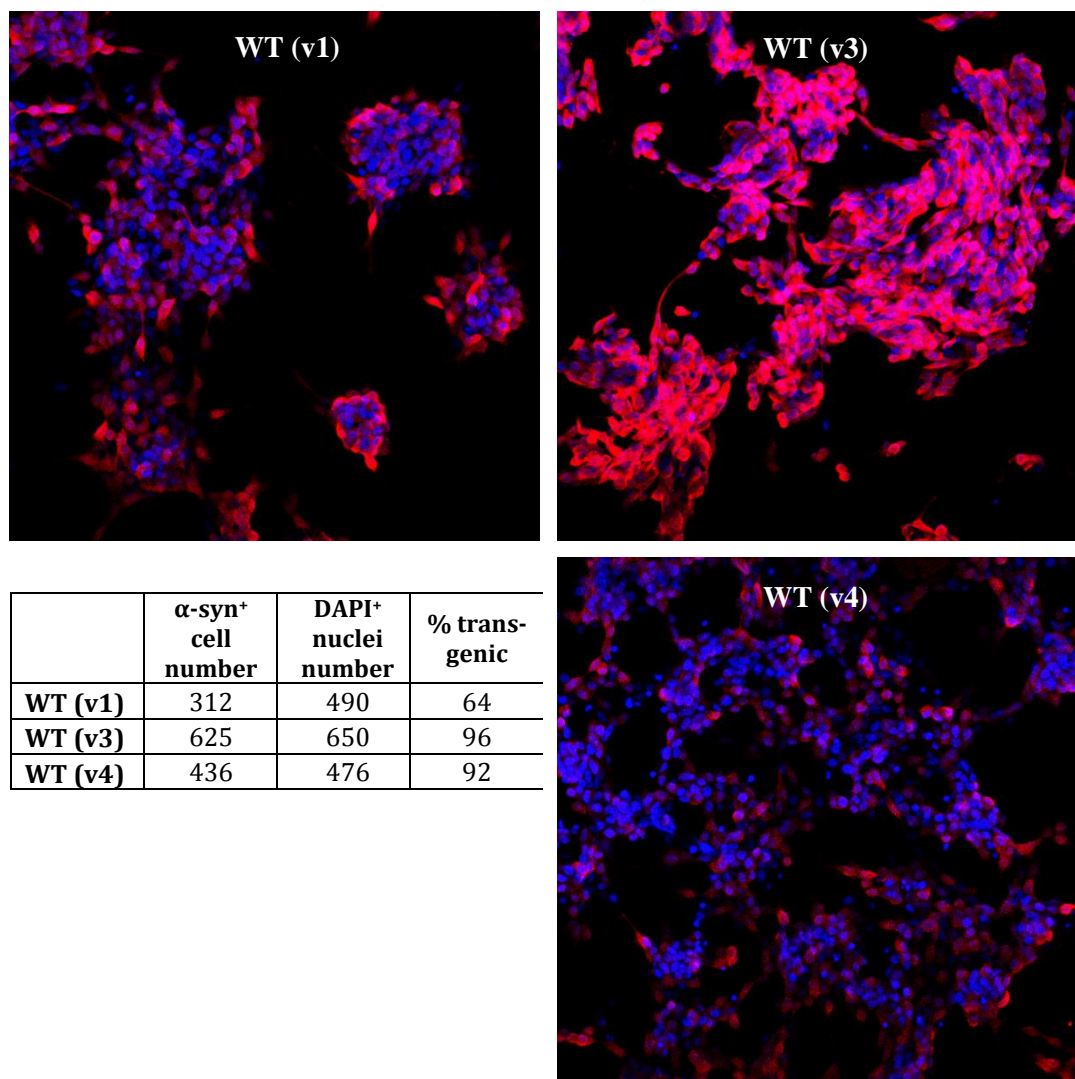


Figure 3.3 Estimated percentage of α -syn-overexpressing cells in three lines of WT α -syn SH-SY5Ys. Immunofluorescent staining for total α -syn was performed, with a DAPI counterstain for the cell nuclei. A single 20x objective view was imaged with an LSM 510 Meta confocal microscope (Zeiss), shown with α -syn (red) and DAPI (blue) staining overlaid. Both the number of α -syn-stained cells and the number of DAPI-stained nuclei were counted separately using the Cell Counter plugin of ImageJ. The percentage of transgenic cells is given as the percentage of α -syn⁺ cells relative to DAPI⁺ nuclei.

3.3.2 α -Syn and APP do not alter one another's expression

A simple way by which two proteins may indirectly interact is through the regulation of protein levels. Changes to transcription, translation, or protein stability can affect protein levels. A previously published study in α -syn SH-SY5Ys found that APP protein expression was increased (Jesko et al. 2014). Therefore, total APP protein levels were measured in α -syn SH-SY5Ys. APP protein expression proved to be unaltered by α -syn (Figure 3.4a). The reverse of this effect was also studied: the impact of APP overexpression upon α -syn protein levels. SH-SY5Ys stably overexpressing the 695 spliced isoform of APP, were generated using a pCI-neo APP695 plasmid construct (Chris Miller, KCL). Levels of α -syn protein were determined by western blotting, and were not affected by APP overexpression (Figure 3.4b).

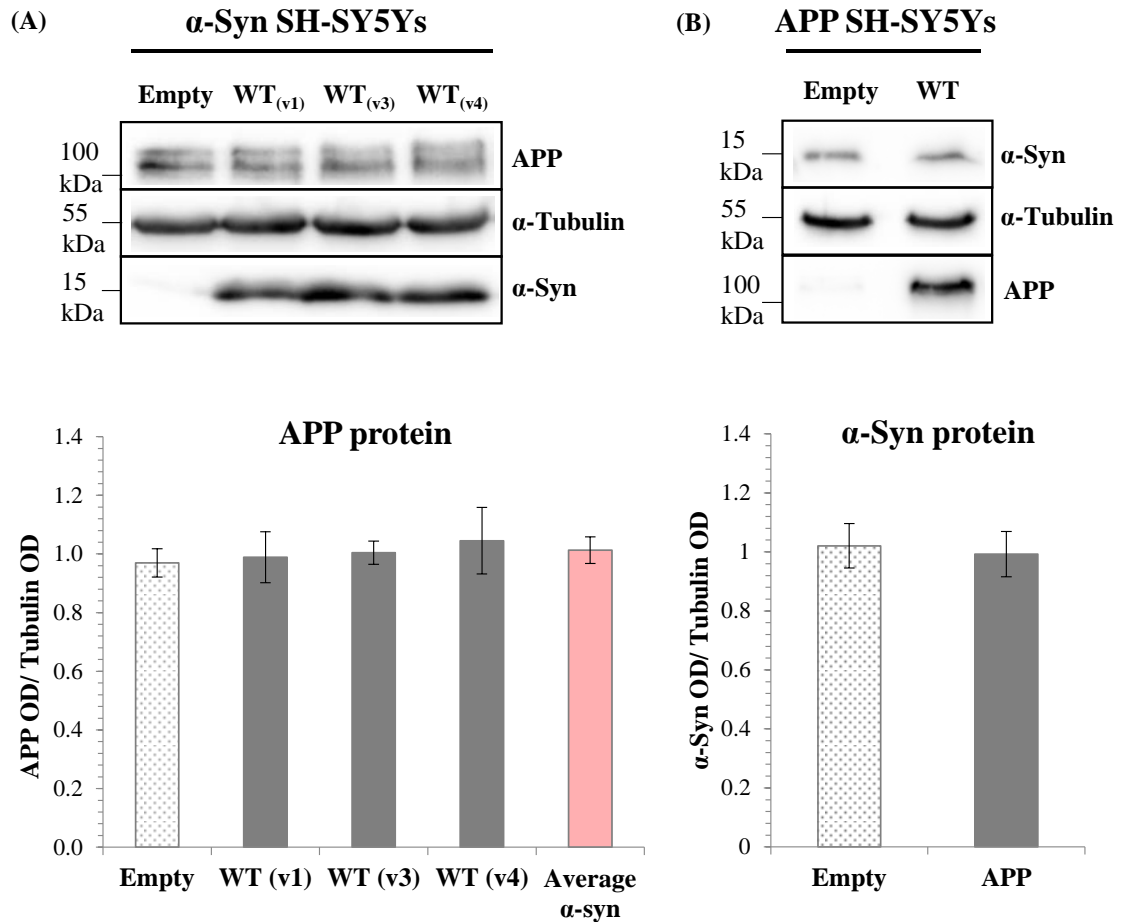


Figure 3.4 α -Syn does not affect APP expression, and APP does not affect α -syn expression in SH-SY5Ys. (A) Western blotting for APP in lysates of three α -syn SH-SY5Y lines. Mean APP OD: α -tubulin OD for 4 independent experiments \pm S.E, and a bar representing an average of the three WT lines. No significant differences between individual WT α -syn lines and the empty vector, calculated by one-way ANOVA with a Tukey HSD post-hoc test. (B) Western blotting for α -syn in lysates of APP SH-SY5Ys. Mean α -syn OD: α -tubulin OD for 12 independent experiments \pm S.E.

3.3.3 α -Syn, but not β -syn, expression increases extracellular secretion of β -amyloid in SH-SY5Ys

The action of β - and γ -secretases on APP within endosomal compartments of a cell releases β -amyloid peptides of variable length. The most common β -amyloid peptide is A β 40, but it is the less abundant A β 42 that has the greatest potential to form toxic aggregates (Hubin et al. 2014). β -Amyloid is secreted, and in cell cultures accumulates in the extracellular medium over a number of days to a detectable level. An ELISA-style kit supplied from Meso Scale Discovery was used to accurately determine A β 40 and A β 42 peptide concentration in conditioned media from cell cultures (Figure 2.3) (Oh et al. 2010).

β -Amyloid secretion was measured in WT (v1) α -syn SH-SY5Ys in the presence and absence of secretase inhibitors (Figure 3.5). Secretase inhibitors were used to validate the extent to which measured β -amyloid reflects β -/ γ -secretase-mediated processing of APP. Other factors can affect levels of extracellular β -amyloid, including amyloid-degrading enzymes. β -/ γ -Secretase-mediated processing of APP is rate-limited by the γ -secretase step, which can be inhibited using the compound DAPT. DAPT incubation with WT α -syn SH-SY5Ys caused strong reductions in A β 40 and A β 42 levels. The sensitivity of the assay to β -secretase inhibition was not tested, due to the short half-life of the peptide inhibitor. α -Secretase contribution to the measured β -amyloid was tested, using the inhibitor TAPI-1. In empty vector and WT α -syn SH-SY5Ys, TAPI-1 treatment appeared to promote A β 40 and A β 42 production. Statistical significance of $p < 0.05$ was achieved only for A β 42 secretion in WT cells. This suggests a mitigating role for α -secretase upon β -amyloid production. Overall, extracellular A β 40 and A β 42 levels clearly demonstrate sensitivity to changes in amyloidogenic processing of APP.

α -Syn overexpression appears to enhance the extracellular secretion of β -amyloid. A β 40 and A β 42 were significantly more concentrated in the media of WT (v1) α -syn compared with empty vector cells (Figure 3.5). On average, A β 40 peptide concentrations were 66 pg/ml in WT α -syn conditioned media, whereas A β 42 was about ten-fold less abundant (6 pg/ml).

In addition to testing α -syn, another member of the synuclein family was included to determine whether it shares the same effect on β -amyloid (Figure 3.5). β -Syn has high homology to α -syn, and some apparent functional redundancy in knockout mouse models (Chandra et al. 2004). A line of SH-SY5Ys transfected with pcDNA 3.1 (+)- β -syn (Wright et al. 2013), was confirmed to overexpress β -syn protein (Figure 3.6). Levels of β -amyloid were unaltered in β -syn SH-SY5Ys. It is likely that β -syn does not share the particular cellular activity of α -syn that influences β -amyloid levels.

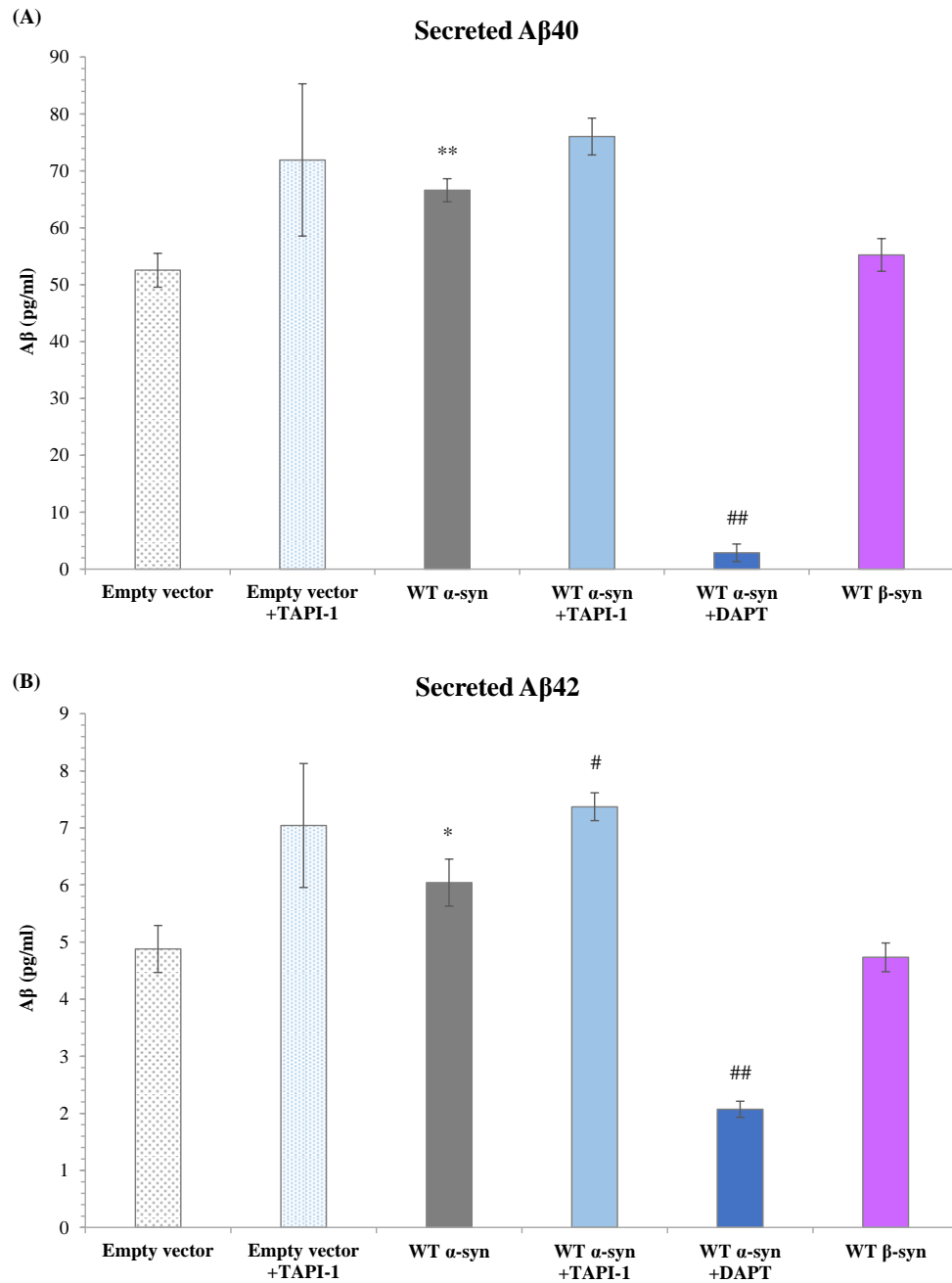


Figure 3.5 WT α -syn expression increases Aβ40 and Aβ42 secretion. (A) Aβ40 and (B) Aβ42 in conditioned media from SH-SY5Ys. Cells were treated with α -secretase inhibitor TAPI-1 (50 μ M) or γ -secretase inhibitor DAPT (10 μ M) for 72 hours. The conditioned media was analysed by Meso Scale Discovery Aβ Peptide Panel 1 (6E10) multiplex assay. Peptide concentrations were calculated by Meso Scale Discovery Workbench software, with reference to a standard curve. Data was normalised by division with the mean peptide concentration of each experiment, and multiplied by the mean of the means. Bar chart represents mean Aβ concentration for 3-4 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector; # $p < 0.05$, ## $p < 0.01$ relative to untreated WT α -syn, calculated by Student t-tests.

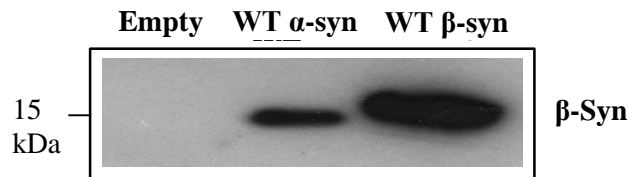


Figure 3.6 β -Syn expression in WT α -syn and WT β -syn SH-SY5Ys. Western blotting was used to test whole cell lysates for β -syn protein by Dr Jennifer McDowall. Staining in the α -syn lane may be due to cross-reactivity of the β -syn antibody with α -syn, due to protein homology.

3.3.4 α -Syn expression enhances the amyloidogenic processing of APP in SH-SY5Ys

The activity of β - and γ -secretases on APP, known as ‘amyloidogenic processing’, generates β -amyloid in cells. An indirect measurement of endogenous β - and γ -secretase activity can be obtained by transfecting cells with the APP-Gal4 luciferase reporter, described in Figure 2.1. Three WT α -syn SH-SY5Y lines were transiently transfected with the APP-Gal4 reporter. Cleavage of the APP-Gal4 reporter was nearly doubled in WT α -syn SH-SY5Y lines, on average ($p < 0.01$, Figure 3.7a). There were no significant differences between the three independent WT lines, determined by one-way ANOVA. Interpretation of the APP-Gal4 reporter signal as ‘amyloidogenic processing’ depends on the Gal4 tag being liberated solely by β - and γ -cleavage. The use of secretase inhibitors in the assay provides some evidence in favour of this interpretation (Figure 3.7b). A γ -secretase inhibitor, DAPT, strongly suppressed cleavage of APP-Gal4, proving that γ -cleavage is indispensable for reporter activity. Neither of two β -secretase inhibitors, ‘ β SI’ and ‘ β -IV’, had a significant impact, but a small reduction in activity is apparent. α -Secretase inhibition, using TAPI-1, significantly upregulated APP-Gal4 cleavage. One likely explanation for this is that inhibiting α -secretase may reduce the competition for APP ‘substrate’ with β -secretase, allowing greater β -cleavage to occur. In conclusion, the APP-Gal4 construct appears to preferentially report β -/ γ -cleavage over α -/ γ -cleavage of APP, and some substrate competition is evident between the α - and β -secretases at the reporter.

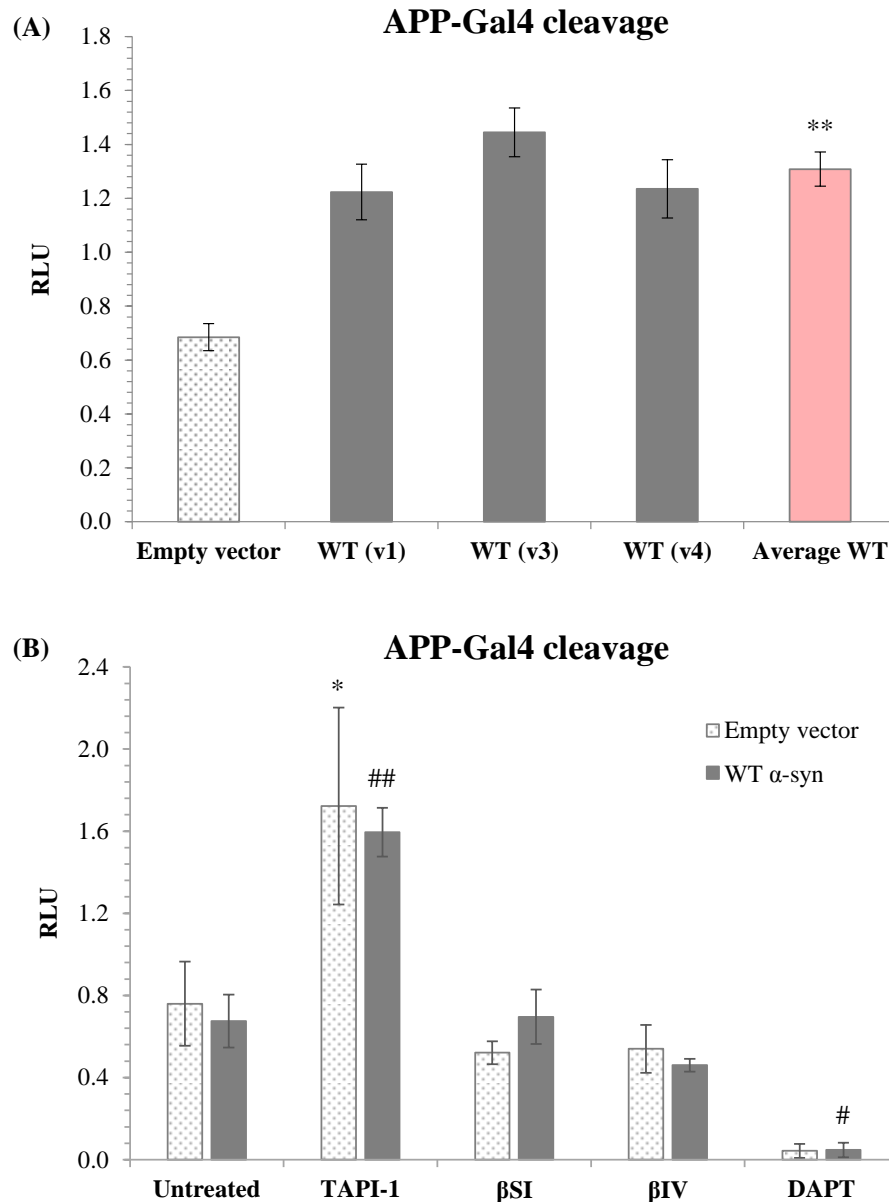


Figure 3.7 WT α -syn expression increases amyloidogenic processing of APP. (A) APP-Gal4 reporter activity is increased in WT α -syn SH-SY5Y lines. Mean RLU for 4 independent experiments \pm S.E, and a bar representing an average of the three WT lines. ** $p < 0.01$ relative to empty vector cells, calculated by Student's t-test. (B) APP-Gal4 assay preferentially reports β -/ γ -secretase processing in empty vector (spotted bars) and WT α -syn SH-SY5Ys (solid bars). Secretase inhibitors and fresh media were given 6 hours post-transfection, and incubated for 16 hours before lysis. Inhibition of α -secretase was with 50 μ M TAPI-1 (EMD Millipore), β -secretase with 10 μ M ' β SI' (Amyloid Precursor Protein β -Secretase Inhibitor, Calbiochem) or 10 μ M ' β -IV' (β -secretase Inhibitor IV, Calbiochem), and γ -secretase with 10 μ M DAPT (Tocris). Mean RLU for 3-6 independent experiments \pm S.E. * $p < 0.05$, relative to (untreated) empty vector; # $p < 0.05$, ## $p < 0.01$ relative to (untreated) WT α -syn, calculated by one-way ANOVA with a Tukey HSD post-hoc test.

Another method of measuring amyloidogenic processing is western blotting for an intermediate APP fragment named 'C99', formed after β -secretase cleavage of APP, but prior to γ -secretase cleavage (Nunan & Small 2000). C99 is the β -cleaved C-terminal fragment (CTF) of APP, of 99 amino acids length, and runs at around 10 kDa on an SDS-PAGE gel. It can only be distinguished from the CTF resulting from α -secretase cleavage, 'C83', by the small difference in SDS-PAGE migration (Figure 2.2). The CTFs accumulate to detectable levels when their degradation by γ -secretase is inhibited, using DAPT. In WT α -syn SH-SY5Y lines, C99 levels were compared either as a ratio of the full length APP protein (Figure 3.8a), or as a ratio of the α -cleavage fragment C83 (Figure 3.8b). In both comparisons WT α -syn cells accumulated higher C99, on average, than empty vector cells ($p < 0.05$). Individual WT α -syn lines showed some variability but are not significantly different to one another, determined by one-way ANOVA. The increased ratio of C99:full-length indicates increased β -secretase cleavage of APP. Yet the effect is weaker when displayed as a ratio of C99:C83. Given that total APP protein levels are known to remain constant, there are two potential explanations for this. Either an overlap in signal between the close C99 and C83 bands blurs any existing differences, or α -secretase cleavage of APP is also enhanced in WT α -syn cells, in addition to β -secretase cleavage.

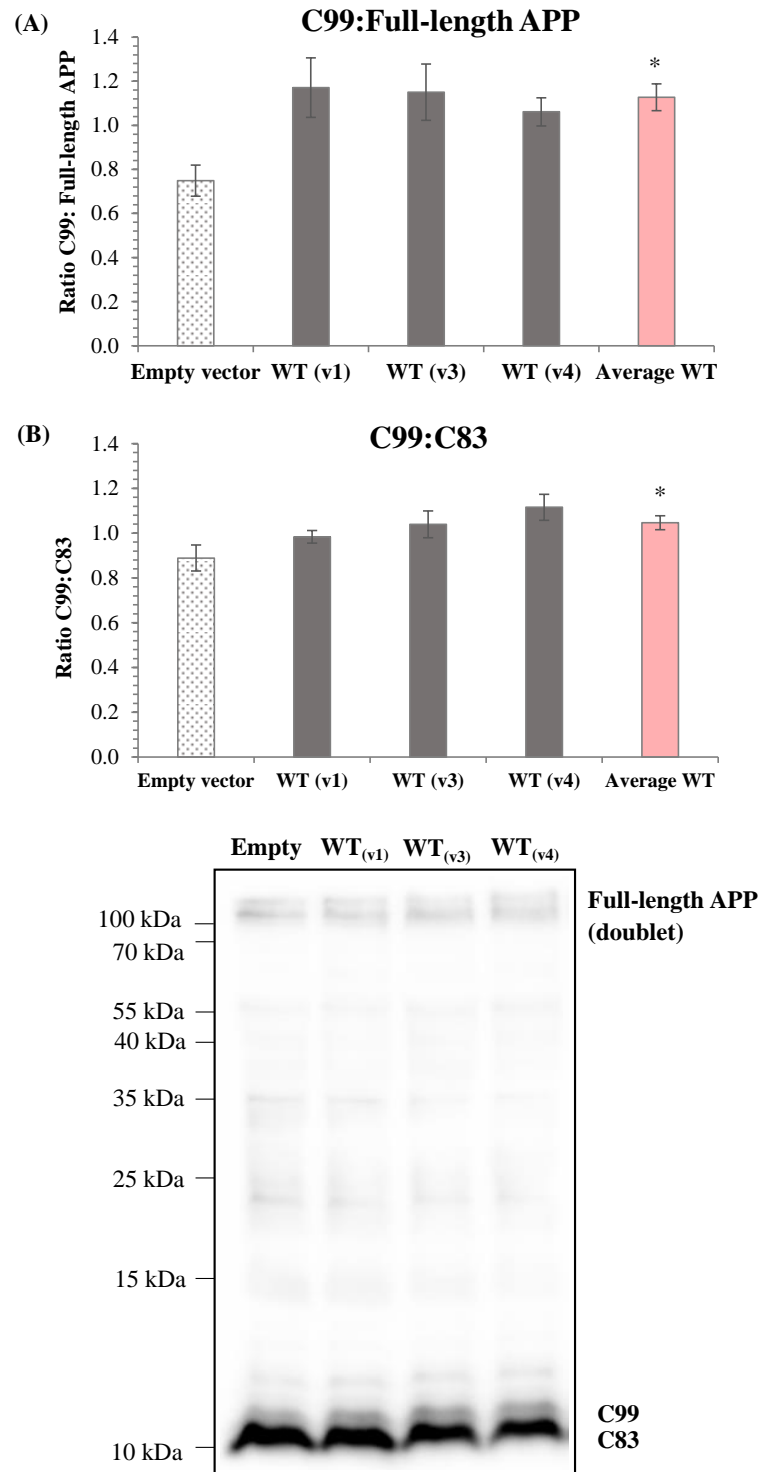


Figure 3.8 Increased β -cleaved APP in WT α -syn SH-SY5Y lines. Western blots of whole-cell lysates were probed with antibody raised against the C-terminus of APP (Y188, Abcam). **(A)** The β -CTF C99 band (~10 kDa) expressed as a ratio to the full-length band (~95 kDa). **(B)** The C99 band (~10 kDa) expressed as a ratio to the α -CTF C83 band (~9 kDa). Representative western blot included. Mean C99 OD: full-length OD, or mean C99 OD: C83 OD, for 4 independent experiments \pm S.E, and a bar representing an average of the three WT lines. * $p < 0.05$ relative to empty vector, calculated by Student's t-test.

3.3.5 Induction of APP amyloidogenic processing by α -syn is replicated in another neuronal cell line N2A, but is not evident in non-neuronal HEK293

Having established that α -syn overexpression in SH-SY5Ys results in enhanced amyloidogenic processing of APP, it was appropriate to investigate the effect in other cell models. HEK293s were chosen as a non-neuronal human cell line, known to express α -syn and APP (Jacobsen & Iverfeldt 2009; Febbraro et al. 2012). N2As were chosen as a neuronal-type mouse cell line. Stably-overexpressing WT α -syn lines were generated with both cell types, and α -syn expression confirmed by western blotting (Figure 3.9a and Figure 3.11a).

WT α -syn HEK293s were initially tested for levels of secreted β -amyloid (Figure 3.9). It was clear that overexpression of α -syn had no effect on the accumulation of extracellular A β 40 and A β 42. Amyloidogenic processing of APP was then assayed using the APP-Gal4 luciferase reporter, as described previously. WT α -syn overexpression significantly reduced amyloidogenic processing (Figure 3.10a). This apparent negative control of amyloidogenic processing by α -syn is a stark contrast to the results obtained in SH-SY5Ys. A mechanism can be suggested from studying the effects of secretase inhibitors (Figure 3.10b). In WT α -syn HEK293s, the activity of the APP-Gal4 reporter is blocked by γ -secretase inhibitor, and increased by α -secretase inhibitor, consistent with reporting of β -/ γ -cleavage of APP. However, in the empty vector HEK293s the higher levels of APP-Gal4 cleavage are not increased by α -secretase inhibition. A likely explanation for the difference is that the ratio of α -cleavage: β -cleavage of APP is lower in empty vector cells than WT α -syn HEK293s. Potentially α -secretase activity may increase with α -syn expression in HEK293s. This would fit better with the data than a decrease in β -secretase activity, because decreased β -secretase activity would not cause cells to become more sensitive to α -secretase inhibition. Overall, the changes have a negligible effect on β -amyloid secretion.

WT α -syn N2As were only assessed for levels of amyloidogenic processing by luciferase reporter assay. Unlike the HEK293s, the N2As appeared to exhibit the same pro-amyloidogenic effect as SH-SY5Ys. WT α -syn N2As showed a robust 1.4-fold increase in amyloidogenic processing relative to empty vector cells (Figure 3.11). The similar results in human and murine neuronal-precursor cells suggest that the effects of α -syn are neuron-specific.

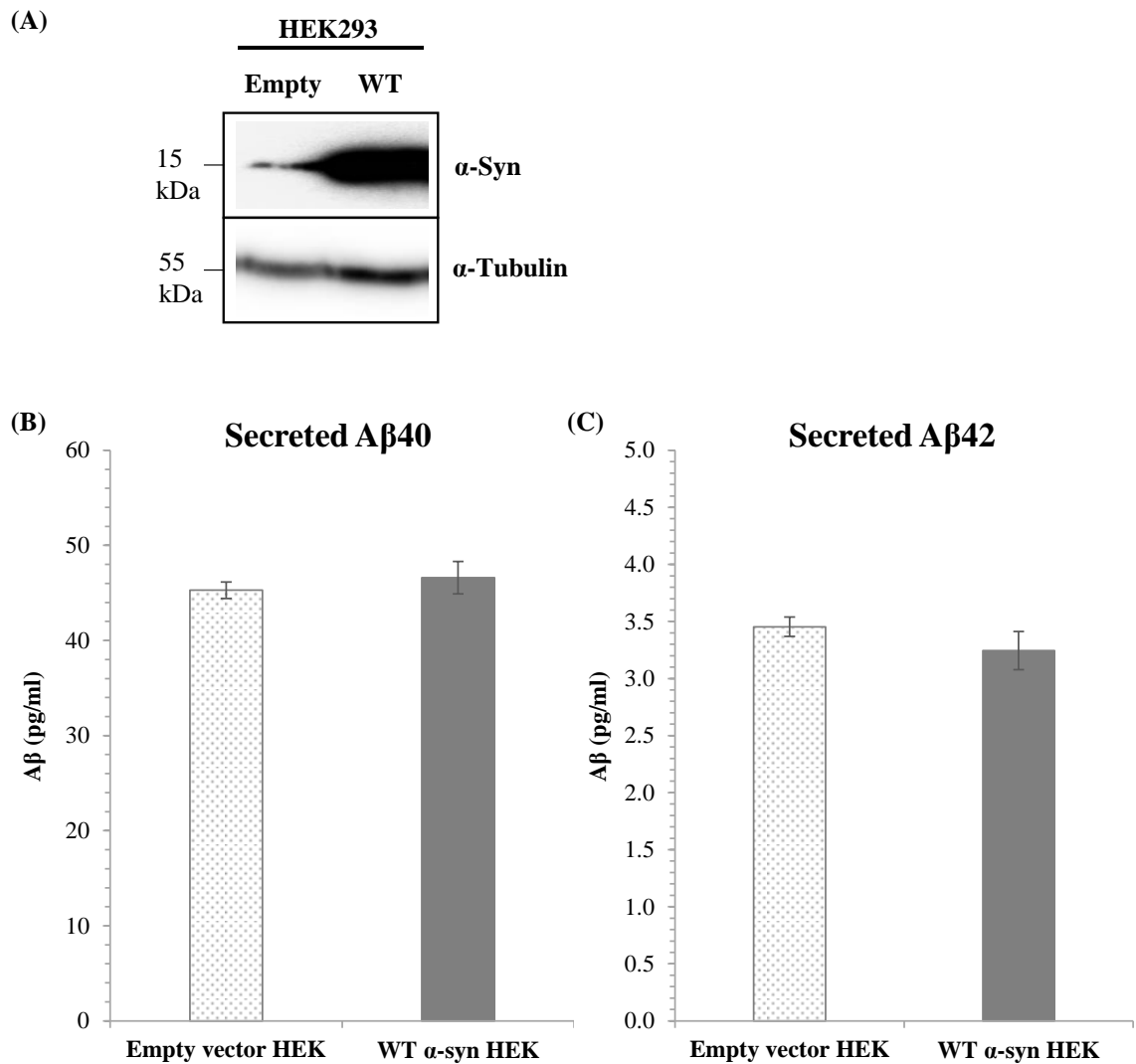


Figure 3.9 A β 40 and A β 42 secretion from HEK293s is not enhanced by WT α -syn overexpression. (A) Overexpression of WT α -syn confirmed by western blotting. Whole cell lysates were tested for α -syn and α -tubulin. (B) Conditioned media from WT α -syn HEK293s was analysed for A β 40 and A β 42 peptides by the Meso Scale Discovery A β Peptide Panel 1 (6E10) multiplex assay. Peptide concentrations were calculated by Meso Scale Discovery Workbench software, with reference to a standard curve. Data was normalised by division with the mean peptide concentration of each experiment, and multiplied by the mean of the means. Bar chart represents mean A β concentration for 3 independent experiments \pm S.E. No significant difference, calculated using a Student's t-test.

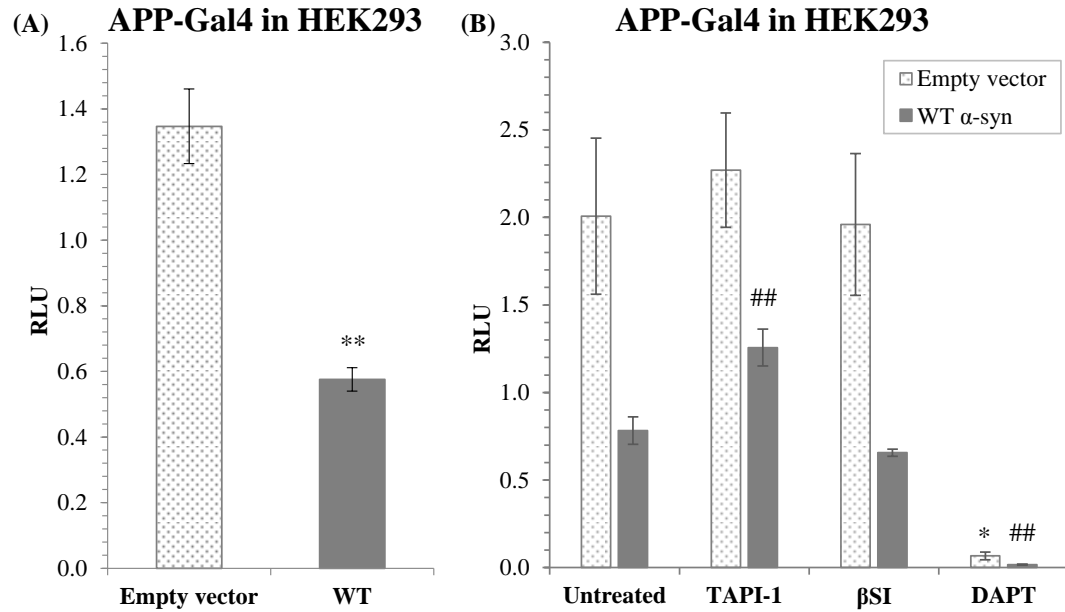


Figure 3.10 Amyloidogenic processing is reduced in WT α -syn HEK293s. (A) APP-Gal4 reporter activity in WT α -syn HEK293s. Mean RLU for 5 independent experiments \pm S.E., Student's t-test. (B) APP-Gal4 assay preferentially reports β -/ γ -secretase processing in WT α -syn HEK293s (solid bars), but this cannot be confirmed in empty vector cells (spotted bars). Secretase inhibitor treatments are detailed in Figure 1.8. Mean RLU for 3 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector, ## $p < 0.01$ relative to untreated WT α -syn, one-way ANOVA with a Tukey HSD post-hoc test.

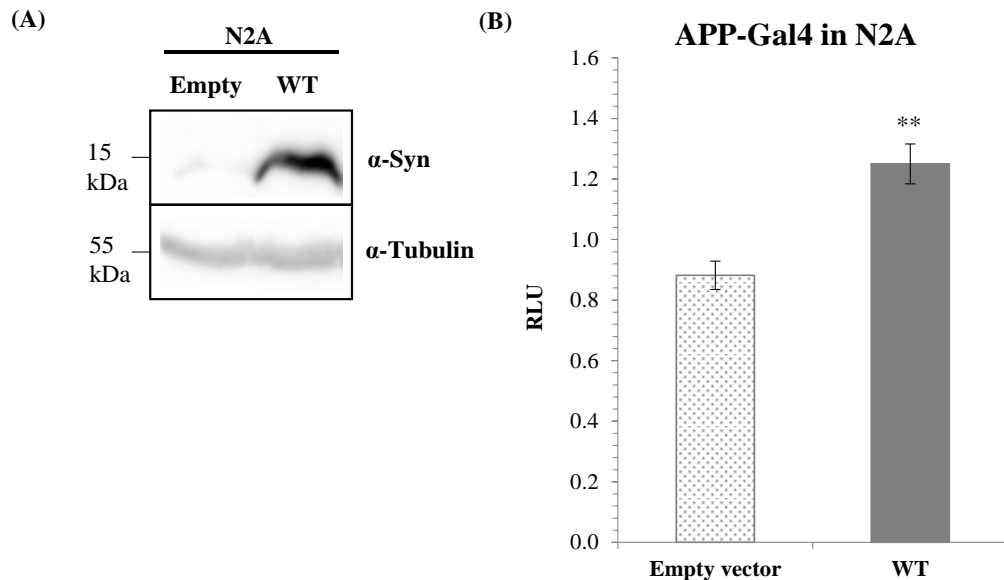


Figure 3.11 Amyloidogenic processing is increased in WT α -syn N2As. (A) Overexpression of WT α -syn confirmed by western blotting. Whole cell lysates were tested for α -syn and α -tubulin. (B) APP-Gal4 reporter activity in WT α -syn N2As. Mean RLU for 5 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

3.3.6 Mutant α -syn SH-SY5Ys have similar expression and subcellular distribution of α -syn to the wildtype lines

To understand better the structural and functional basis by which α -syn mediates an effect on APP processing, a number of pcDNA 3.1(+) - α -syn plasmids with genetic mutations were purified from existing bacterial stocks (supplied by Prof. David Brown), and stably transfected into SH-SY5Ys. Δ 2-9, A53T, and E46K α -syn plasmids were as previously described (Wang et al. 2010). The Δ NAC mutant was generated previously by site-directed mutagenesis of pcDNA3.1(+)- α -syn, using the primer sequences 5'-CAAATGTTGGAGGAGCAGTGGA-GGGAGCAGGGAGCA-3' and 5'-TGCTCCCTGCTCCCTCCACTGCTCCTCCAACATT-TC-3'. Levels of α -syn protein expression in mutant lines were monitored periodically, and did not significantly differ from the WT (v1) line on average (Figure 3.12).

The effect of these mutations on the subcellular distribution and aggregation of α -syn was of interest, to provide extra information on the functional status of α -syn in these cells. As noted in the chapter introduction, mutations in the N-terminus may alter the membrane-binding properties or synaptic targeting of α -syn. Strong alterations to α -syn subcellular localisation can be identified under a confocal microscope with immunofluorescent staining. Immunofluorescent staining of α -syn was performed on the wildtype and mutant α -syn SH-SY5Ys. Changes to the cellular distribution of α -syn were determined qualitatively, and representative images shown in Figure 3.14. Aggregates of mutant α -syn in SH-SY5Ys. WT α -syn cells are distinctly ringed by a bright band of α -syn close to the plasma membrane, in addition to diffuse cytoplasmic staining. This juxtamembrane staining is repeated in all of the mutants except for Δ 2-9 α -syn cells, which appear to have only a homogenous cytoplasmic stain. This suggests that the Δ 2-9 α -syn SH-SY5Ys may have altered subcellular distribution. Confirmation of this would require other methods, as strong α -syn overexpression itself could saturate membranes and appear to increase cytoplasmic staining. Additionally, subtle changes to α -syn subcellular localisation cannot be detected by this method.

The presence of insoluble intracellular aggregates of α -syn should also be highlighted by immunofluorescent staining. Examination of all three WT α -syn lines did not reveal any strongly uneven staining (data not shown). SH-SY5Y lines expressing the disease-associated point mutants E46K and A53T were scrutinised for any evidence of bright inclusions, with twenty 63x confocal microscope images of each line. No completely unequivocal inclusions were identified. Figure 3.14 shows selected images where E46K and A53T SH-SY5Ys have some bright patches of α -syn staining. However, these are unlikely to be genuine α -syn aggregates, given their diffuse edges and rarity. More likely the brighter staining represents narrow protrusions of the plasma membrane, with which α -syn associates.

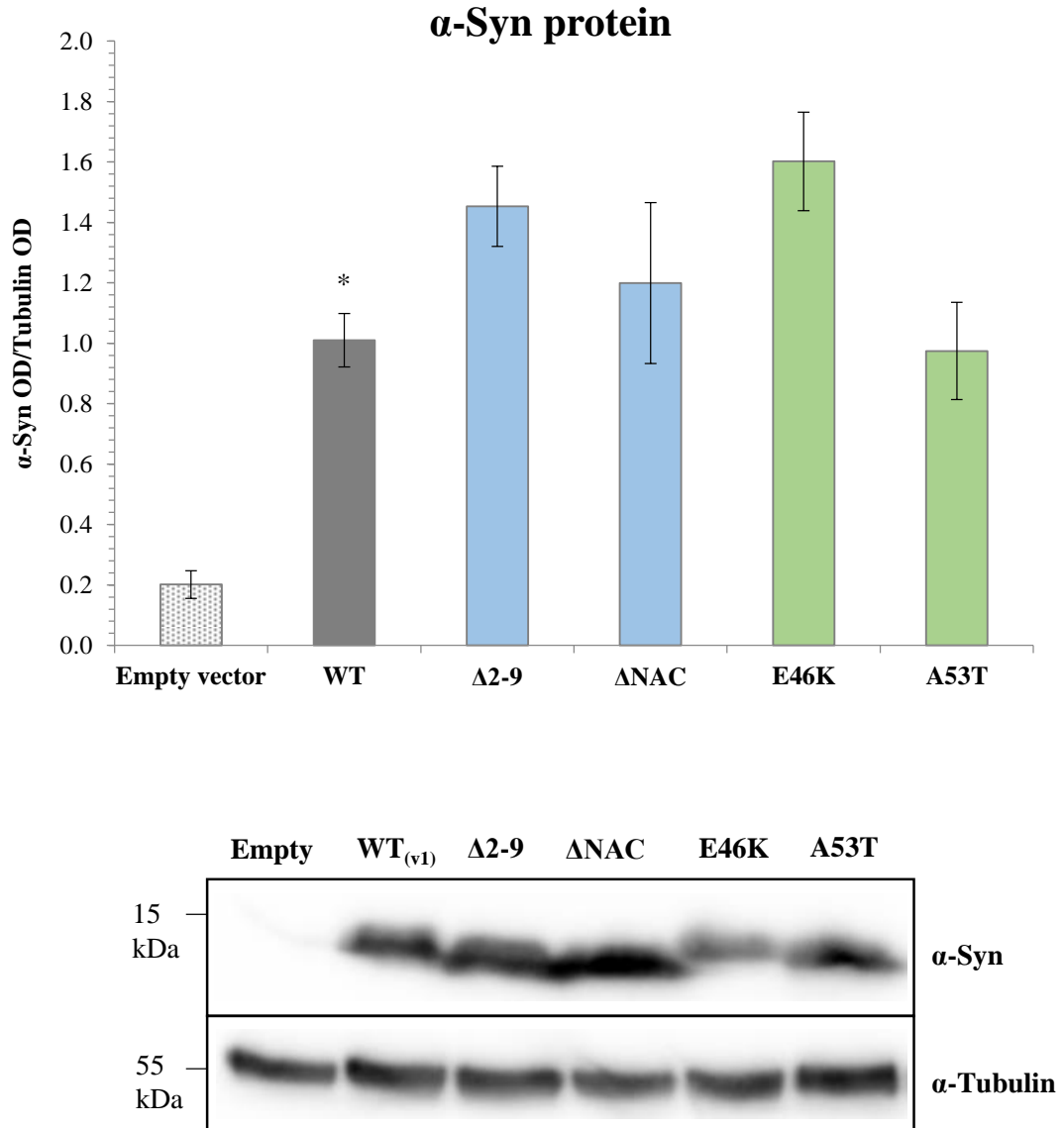


Figure 3.12 α -Syn protein levels in mutant α -syn SH-SY5Y lines. Whole cell lysates were tested for α -syn and α -tubulin by western blotting. Mean α -syn OD: α -tubulin OD for 7 independent experiments \pm S.E.* $p < 0.05$ relative to empty vector, no significant difference between mutants and WT α -syn, calculated by pairwise t-tests with a Holm adjustment.

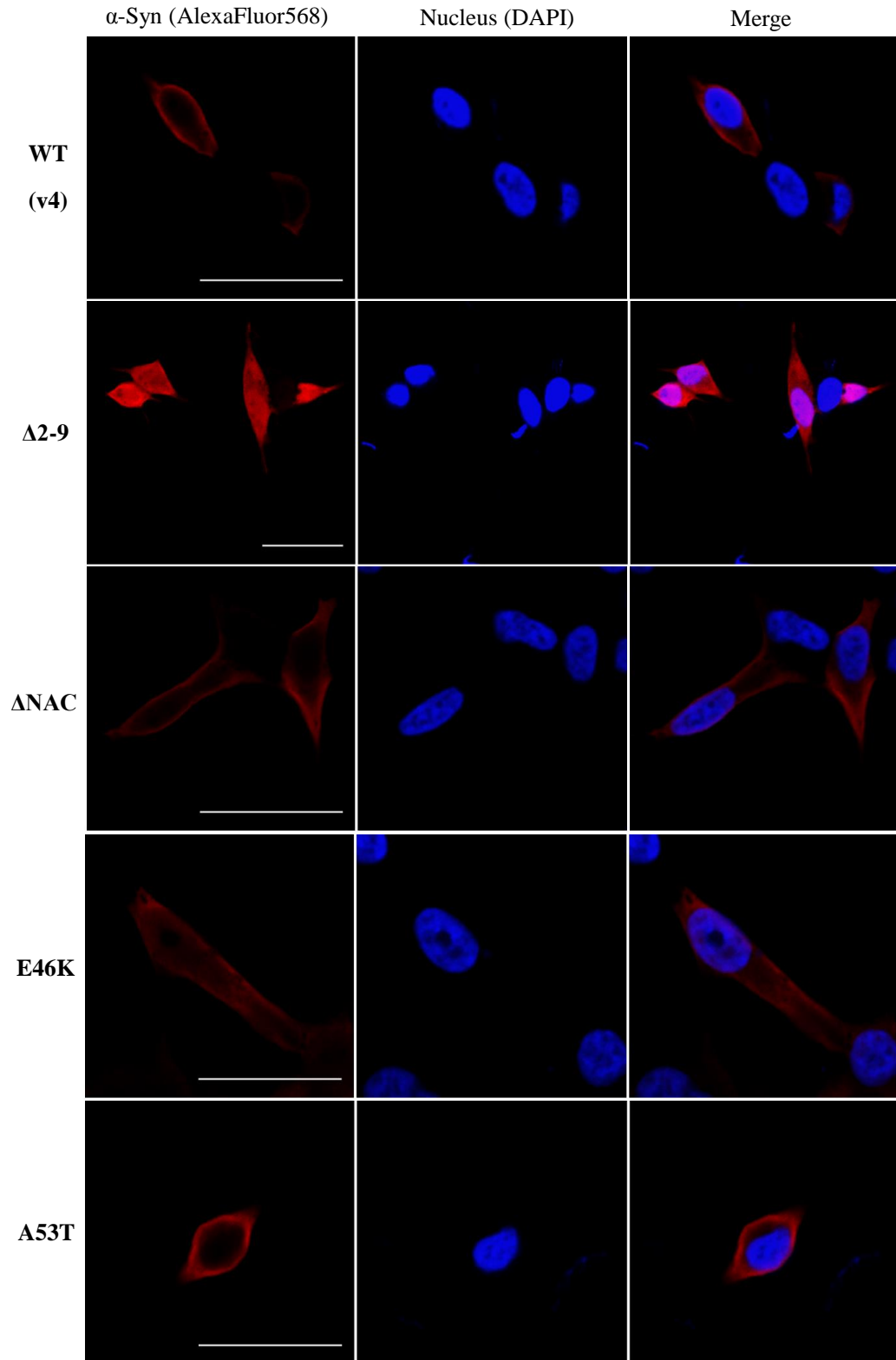


Figure 3.13 Distribution of mutant α -syn in SH-SY5Ys. α -Syn immunofluorescent staining in WT, Δ 2-9, Δ NAC, E46K, and A53T α -syn SHSY5Ys (representative images). Images were viewed with a 63x objective (Zeiss Anti-Flex Plan-Neofluar x63 /1.25 Oil Ph3) on an LSM 510 Meta confocal microscope (Zeiss). Scale bar: 25 μ m.

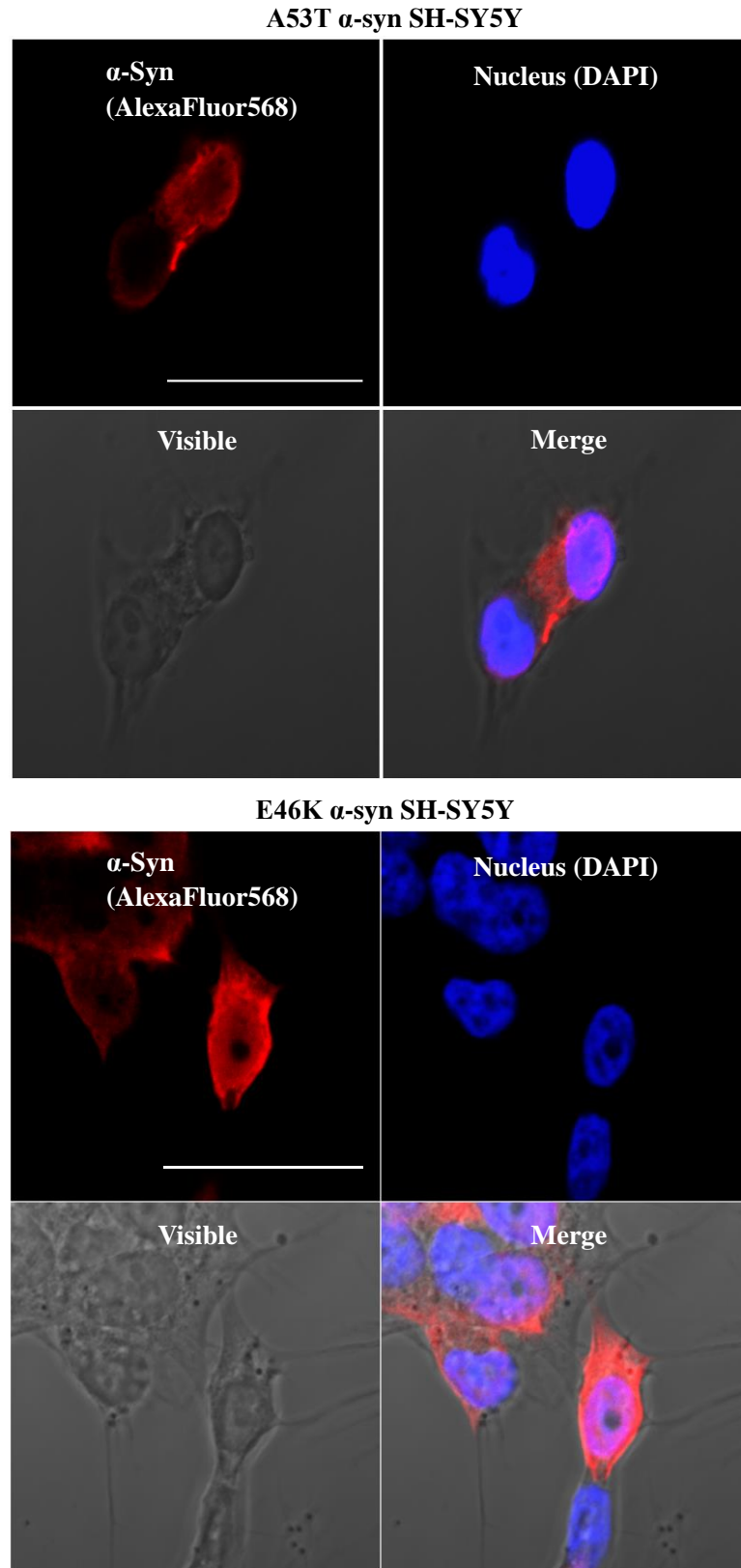


Figure 3.14 Aggregates of mutant α -syn in SH-SY5Ys. Irregular α -syn immunofluorescent staining in E46K and A53T α -syn SHSY5Ys (not representative of most images). Images were viewed with a 63x objective (Zeiss Anti-Flex Plan-Neofluar x63 /1.25 Oil Ph3) on an LSM 510 Meta confocal microscope (Zeiss). Scale bar: 25 μ m.

3.3.7 Specific mutations of α -syn enhance β -amyloid secretion when over-expressed in SH-SY5Ys

β -Amyloid secretion was measured in SH-SY5Y lines overexpressing mutant α -syn (Figure 3.15). Two N-terminal truncations, Δ 2-9 and Δ NAC, were used to ascertain whether the effect of α -syn on β -amyloid is influenced by N-terminal domain structure. A dominant-negative loss of function was predicted, potentially resulting in decreased β -amyloid secretion. Interestingly, Δ 2-9 and Δ NAC did not decrease secreted β -amyloid levels, relative to the WT (v1) line. In fact there was 3-fold *higher* β -amyloid levels in the conditioned media of Δ 2-9 α -syn SH-SY5Ys. No significant change to β -amyloid was evident in the Δ NAC α -syn cells. Additionally, β -amyloid secretion was measured in cells with the disease-associated point mutations E46K or A53T. In E46K cells, secreted β -amyloid was significantly elevated by 1.25-fold. A53T had no significant effect, perhaps surprising given its relatively close sequence proximity to E46K. β -Amyloid secretion can clearly be potentiated by mutated forms of α -syn, but the confinement of this effect to particular unrelated mutations does not suggest an obvious mechanism.

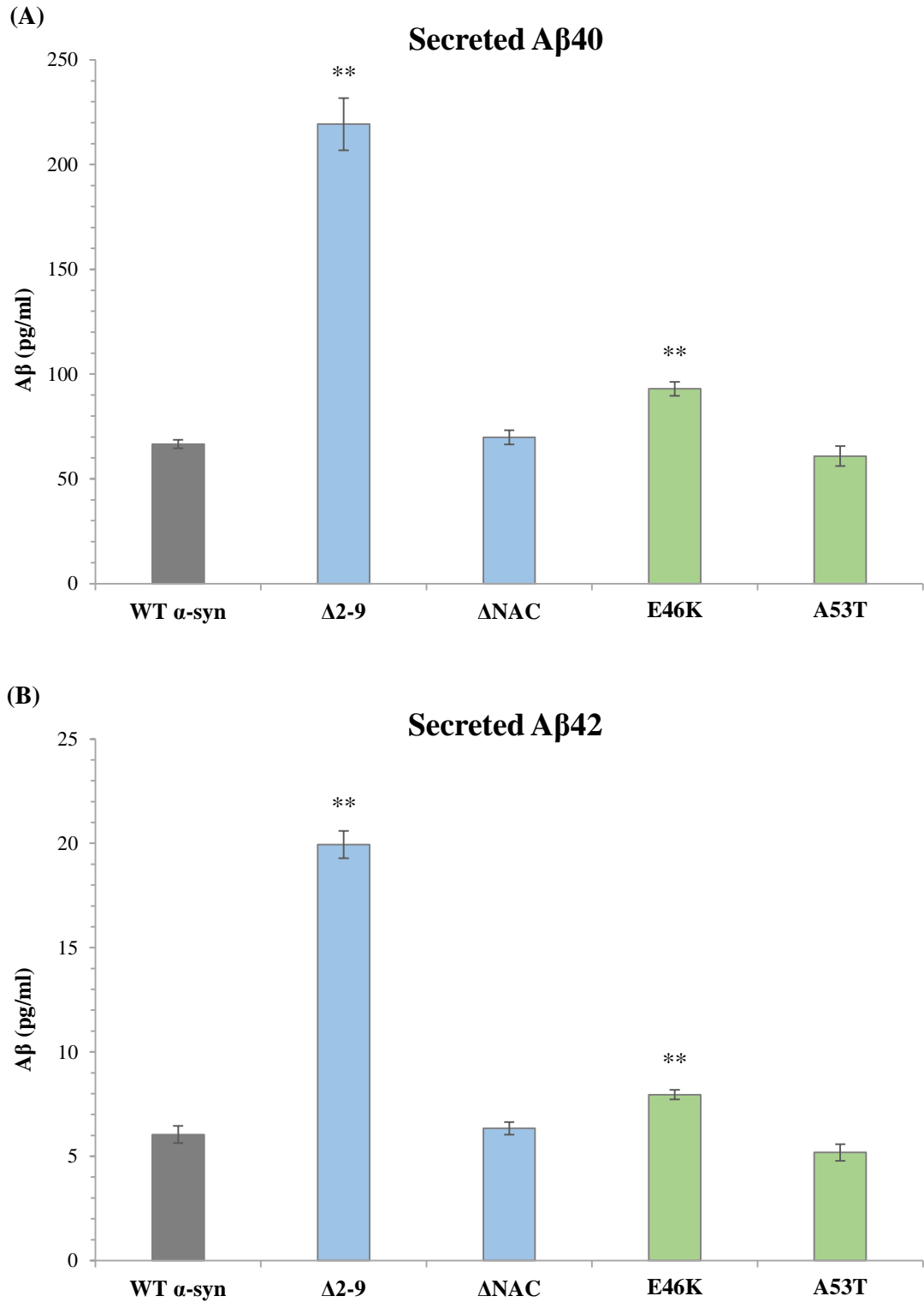


Figure 3.15 Specific mutants of α -syn increase A β 40 and A β 42 secretion.(A) A β 40 and (B) A β 42 in conditioned media from SH-SY5Ys. The conditioned media was analysed by Meso Scale Discovery A β Peptide Panel 1 (6E10) multiplex assay. Peptide concentrations were calculated by Meso Scale Discovery Workbench software, with reference to a standard curve. Mean A β concentration for 3-6 independent experiments \pm S.E. ** p <0.01 relative to empty vector calculated by pairwise t-tests with a Holm adjustment.

3.3.8 Specific mutations of α -syn modulate the amyloidogenic processing of APP when over-expressed in SH-SY5Ys

Amyloidogenic processing of APP was investigated in mutant α -syn SH-SY5Ys, using the aforementioned APP-Gal4 luciferase reporter assay. In comparison to the β -amyloid measurements, a wider selection of mutants were screened for changes to amyloidogenic processing by this method. In addition to the truncation mutants Δ 2-9 and Δ NAC, and disease mutants E46K and A53T, a third disease-associated point mutation was screened, A30P (Wang et al. 2010). Two mutations of a phosphorylation site at S129 were also included, S129A and S129D. S129A was generated previously by site-directed mutagenesis of pcDNA3.1(+)- α -syn, using the primers 5'-GAAATGCCTGCTGAGGAAGGG-3' and 5'-CCCTTCCTCAGCAGGCATTTC-3'. S129D was generated using the primers 5'-GAAATGCCTGATGAGGAAGGG-3' and 5'-CCCTTCCTCATCAGGCATTTC-3'.

N-terminal truncation mutant Δ 2-9 had a strong potentiating effect on APP-Gal4 cleavage, as did the other truncation mutant Δ NAC (Figure 3.16). The strong effect of Δ 2-9 on APP-Gal4 cleavage, a 7-fold increase over WT, mirrors its 3-fold potentiation of β -amyloid secretion. However, the robust 3-fold upregulation of APP-Gal4 cleavage in Δ NAC does not reflect the unaltered β -amyloid levels measured in this line.

The disease-associated point mutations, A30P, A53T, and E46K, all slightly enhanced APP-Gal4 cleavage, but this is only statistically significant for A53T (Figure 3.16). Given the subtlety of the change, one cannot be certain that the increase in APP-Gal4 cleavage is not influenced by varying expression levels of α -syn. The disease-mutant line that significantly increased APP-Gal4 cleavage (A53T) is not the same one that significantly increased β -amyloid secretion (E46K).

Phosphorylation site mutations of Ser-129 were also examined, since the block (S129A) or mimicry (S129D) of Ser-129 phosphorylation is thought to have opposing effects on the toxicity of α -syn. S129A slightly reduced APP-Gal4 cleavage ($p < 0.05$) compared with the wildtype, whereas S129D had little effect (Figure 3.16). The reduced APP-Gal4 cleavage in S129A cells is significant in isolation. Significance of all individual mutations was calculated with unadjusted Student's t-tests, since they were largely assayed separately. However the S129A and S129D mutations were compared simultaneously with the wildtype, so analysis of variance would be appropriate. ANOVA testing with post-hoc Tukey HSD shows no significant effect of S129A/D compared with WT, yet the difference in APP-Gal4 cleavage between the two phosphorylation mutants is significant ($p < 0.05$). The implication is that Ser-129 phosphorylation could subtly potentiate the effect of α -syn on APP-Gal4 cleavage.

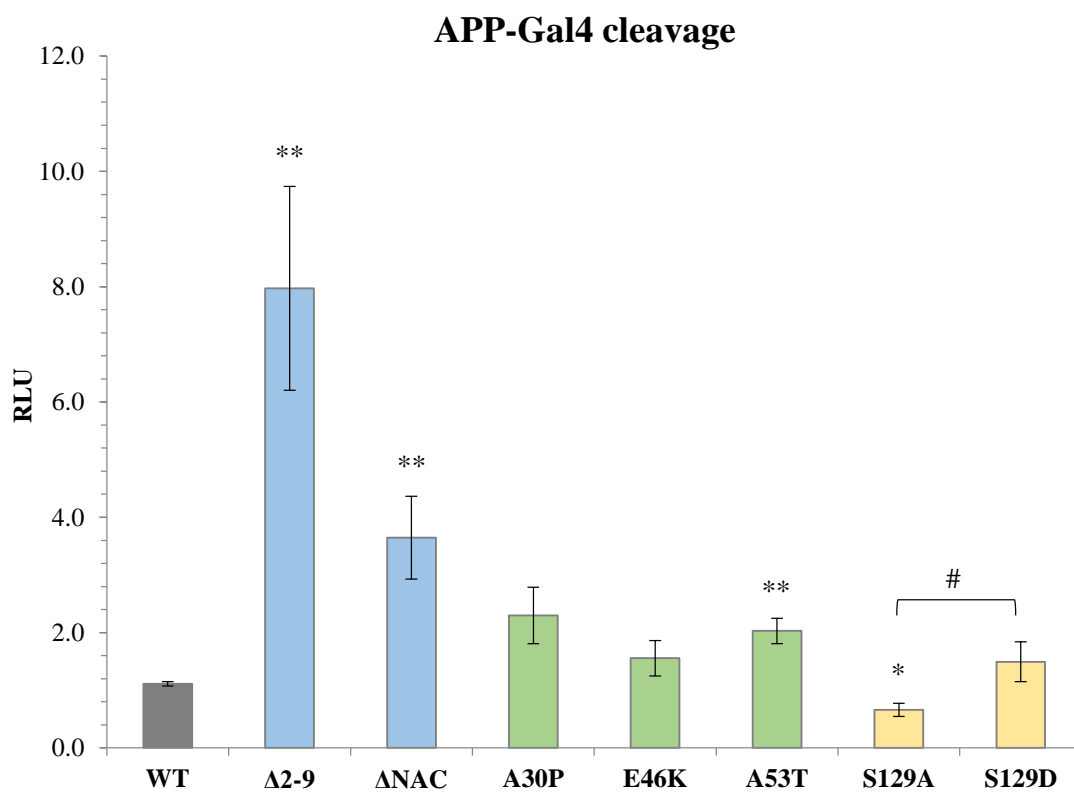


Figure 3.16 APP-Gal4 reporter activity in mutant α -syn SH-SY5Y lines. Mean RLU for 3-5 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to WT α -syn, calculated by Student's T-test. # $p < 0.05$ between S129 mutants, calculated by one-way ANOVA with a post-hoc Tukey HSD test.

Amyloidogenic processing was also studied for several of the mutant lines with a different technique, measuring the intracellular generation of APP C-terminal fragments, C99 and C83. As previously, these were detected by western blotting for APP in extracts of cells treated with a γ -secretase inhibitor, causing CTFs to accumulate. The effect of wildtype α -syn had been to enhance C99 production (β -cleavage) as a ratio of full-length protein, and increase the ratio of C99 to C83 (α -cleavage). CTFs were measured in Δ 2-9, Δ NAC, E46K, and A53T. Only E46K significantly enhanced C99 production as a ratio of full-length protein (Figure 3.17a). A marked difference between the mutants and WT (v1) line emerges when C99 levels are expressed as a ratio of C83. When compared with C83, C99 levels were significantly higher in Δ NAC, E46K and A53T α -syn SH-SY5Ys than the wildtype line (Figure 3.17b). Curiously, there is little variation between the mutants, compared with the differences in APP-Gal4 cleavage. In particular one would expect Δ 2-9 α -syn cells have greater C99 levels than the other mutants. Potentially the level to which C99 fragments can accumulate under γ -secretase inhibition may be limited, by degradation or negative feedback processes, creating a ceiling that erases differences between the mutant lines. Full-length APP protein levels are not altered by mutant α -syn overexpression (Figure 3.18), as demonstrated previously with the WT lines, so the changes to CTF production are likely reflect altered APP processing.

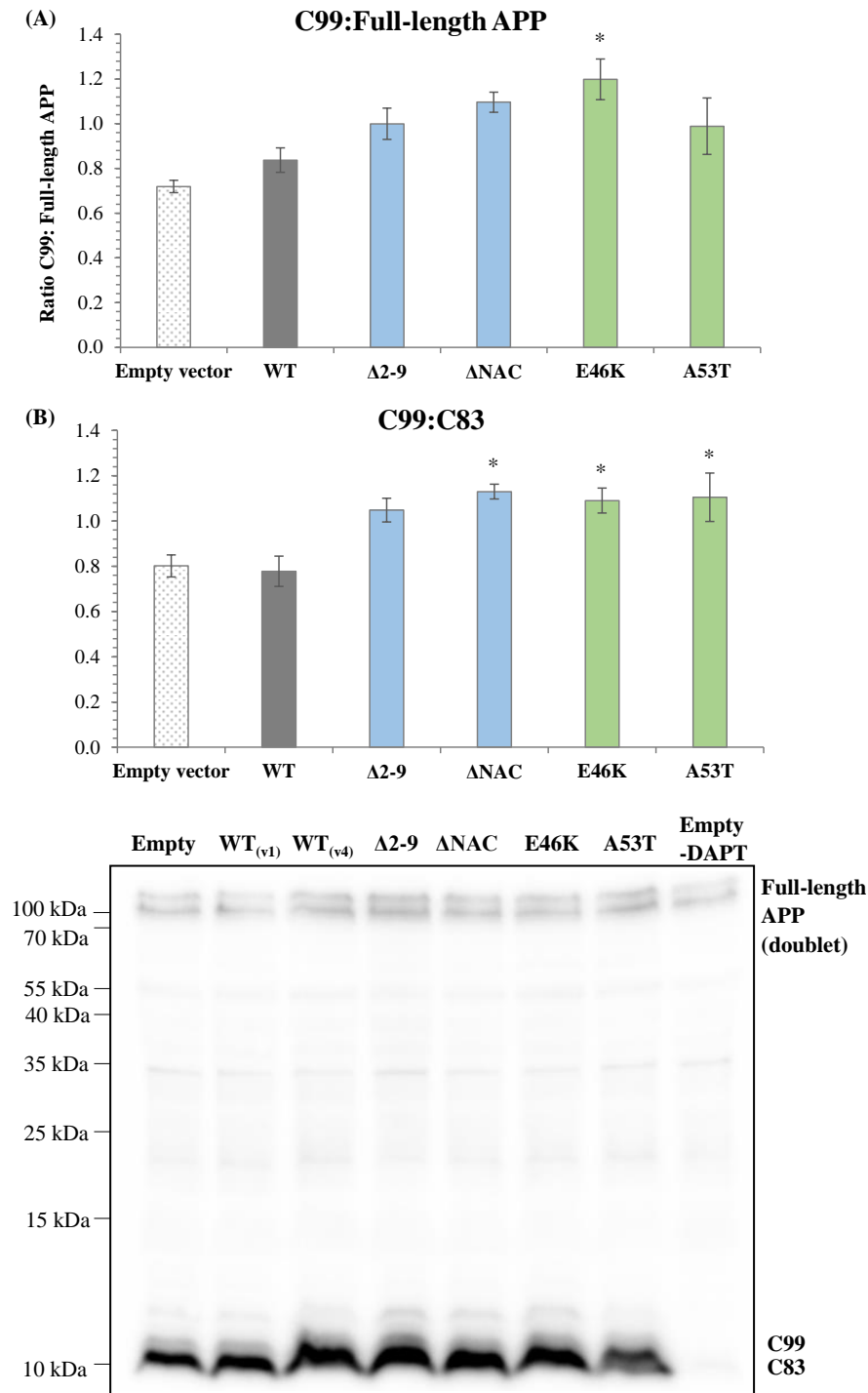


Figure 3.17 Increased β -cleaved APP in mutant α -syn SH-SY5Y lines. Western blots of whole-cell lysates were probed with antibody raised against the C-terminus of APP. **(A)** The β -CTF C99 band (~10 kDa) expressed as a ratio to the full-length band (~95 kDa). **(B)** The C99 band (~10 kDa) expressed as a ratio to the α -CTF C83 band (~9 kDa). Representative western blot shown, including a negative control from empty vector cells not treated with DAPT. Mean C99 OD: full-length OD, or mean C99 OD: C83 OD, for 4 independent experiments \pm S.E. * $p < 0.05$ relative to WT (v1) α -syn, calculated by one-way ANOVA with a Tukey HSD post-hoc test.

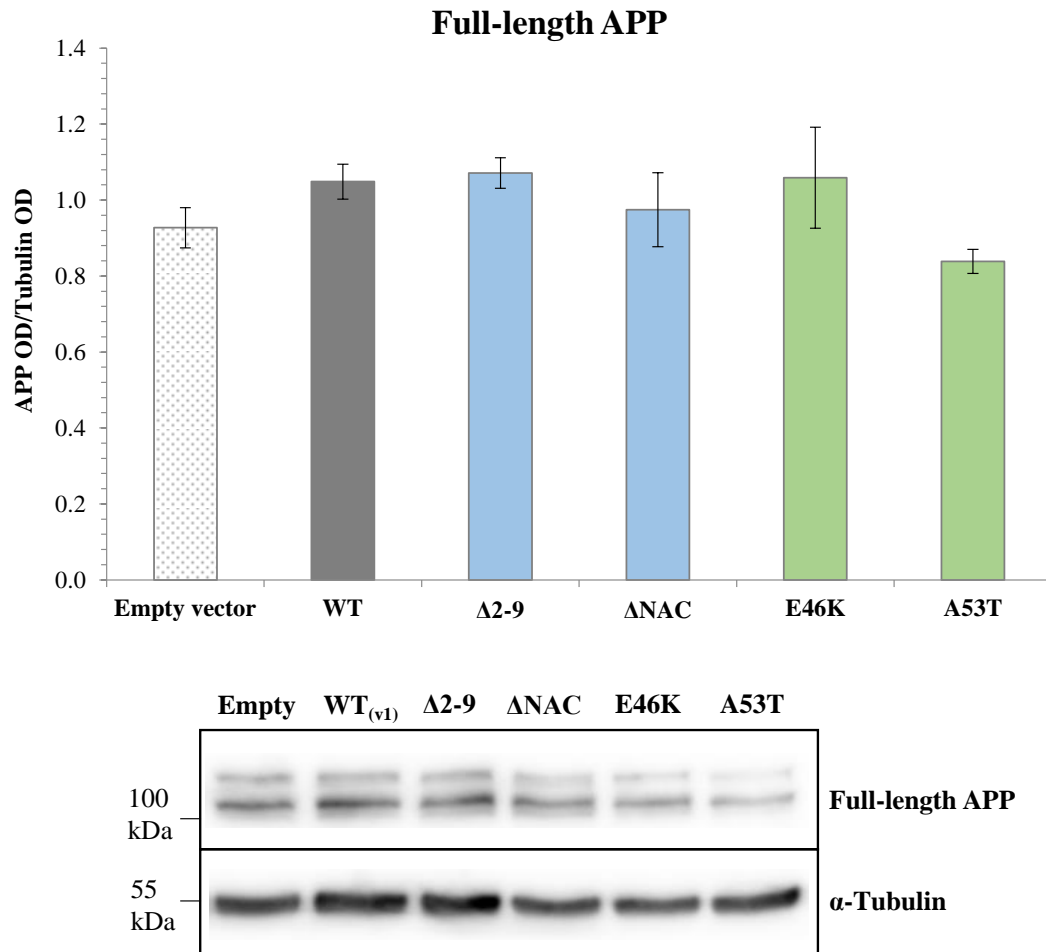


Figure 3.18 Levels of full-length APP protein are unaltered in mutant α -syn SH-SY5Ys. Western blots of whole-cell lysates were probed with antibody raised against the C-terminus of APP. Mean APP OD: α -tubulin OD for 4 independent experiments \pm S.E. No significant differences between individual mutant α -syn lines and the WT, calculated by one-way ANOVA with a Tukey HSD post-hoc test.

3.3.9 *α -Syn mutant protein modulates the amyloidogenic processing of APP in N2As and HEK293s*

The potentiating effect of wildtype α -syn upon APP amyloidogenic processing was previously shown to be cell-type specific, occurring in neuron-like SH-SY5Y and N2A cell lines, but not in the fibroblast-like HEK293. Having established differential effects of α -syn mutations in transgenic SH-SY5Ys, it was hypothesised that a similar pattern could be detected in the mouse neuronal cell line N2A, and perhaps a different effect in non-neuronal HEK293s. Two mutations were selected: the Δ 2-9 truncation because it most strongly enhanced amyloidogenic processing in SH-SY5Ys, and the disease-associated mutation E46K, which had a weaker effect on amyloidogenic processing but still enhanced β -amyloid generation. Δ 2-9 and E46K were stably transfected into N2A cells and HEK293 cells, and α -syn expression confirmed by western blotting (Figure 3.19a, Figure 3.20a).

Mutant HEK293 lines appear to have comparable α -syn expression to the WT α -syn line (Figure 3.19a). Previously it was shown that WT α -syn has an anti-amyloidogenic effect on APP processing in HEK293 cells, the opposite effect to neuronal cells. Interestingly, the two α -syn mutants appear to change APP-Gal4 cleavage in the pro-amyloidogenic direction (Figure 3.19b). The Δ 2-9 mutation had limited effect on APP-Gal4 and is not significantly different to the WT. Yet the E46K mutant strongly enhanced APP-Gal4 cleavage, i.e. abolishing the anti-amyloidogenic effect of WT α -syn in HEK293 cells. Indeed there is no significant difference in APP-Gal4 cleavage between E46K and the empty vector (not shown). A better mechanistic understanding would be needed to determine whether E46K reduces a signal by α -syn or introduces a new opposing signal.

In the two mutant N2A lines, α -syn expression also dissimilar to the WT. Levels of α -syn were significantly lower in Δ 2-9 N2A, and significantly higher in E46K N2A (Figure 3.20a). This needs to be taken into account when interpreting amyloidogenic effects. It was hypothesised that amyloidogenic processing would be strongly potentiated by Δ 2-9, and weakly potentiated by E46K, which was the effect in SH-SY5Ys. In contrast with the SH-SY5Y data, the Δ 2-9 mutant N2As did not significant enhance amyloidogenic processing relative to WT α -syn N2As (Figure 3.20b). The E46K N2As appeared to strongly upregulate APP cleavage. It is important to note that APP-Gal4 reporter activity in the α -syn lines closely mirrors α -syn expression levels. No conclusions can therefore be made about the impact of mutant α -syn in N2As.

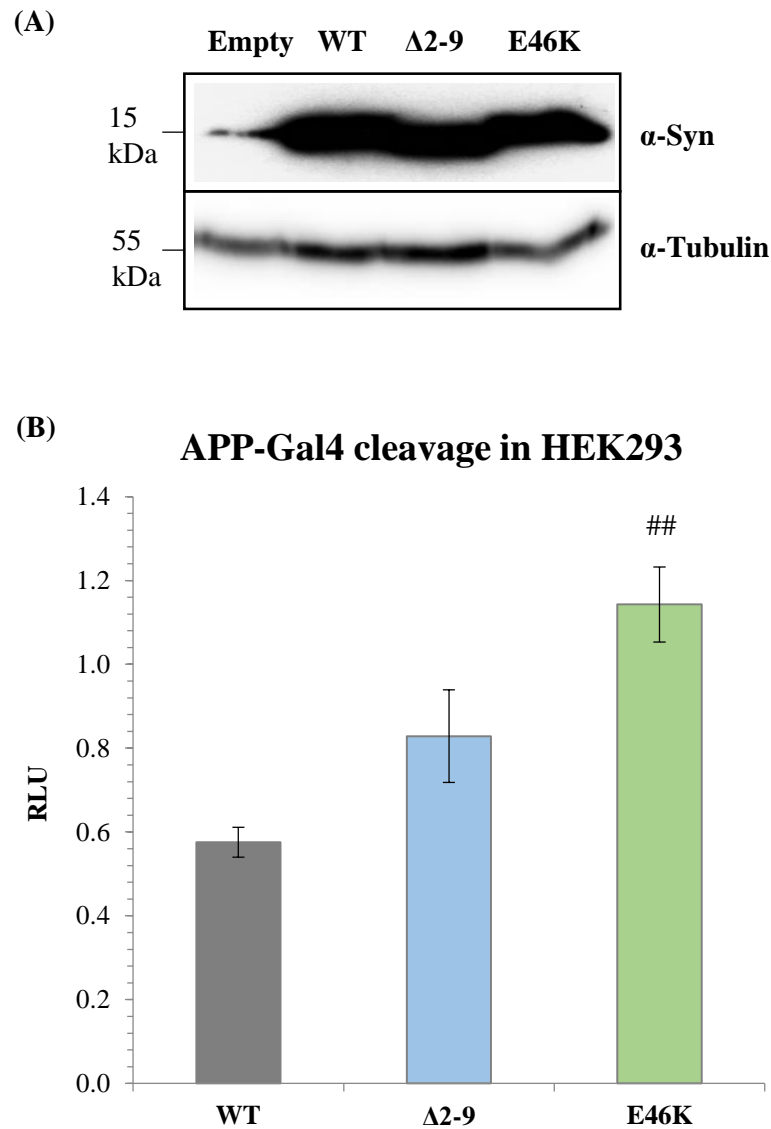


Figure 3.19 Amyloidogenic processing is increased in E46K α -syn HEK293s. (A) Overexpression of α -syn confirmed by western blotting. Whole cell lysates were tested for α -syn and α -tubulin. A representative western blot is shown. (B) APP-Gal4 reporter activity in mutant α -syn HEK293s. Mean RLU for 5 independent experiments \pm S.E. ## $p < 0.01$ relative to WT, calculated by pairwise t-tests with a Holm adjustment.

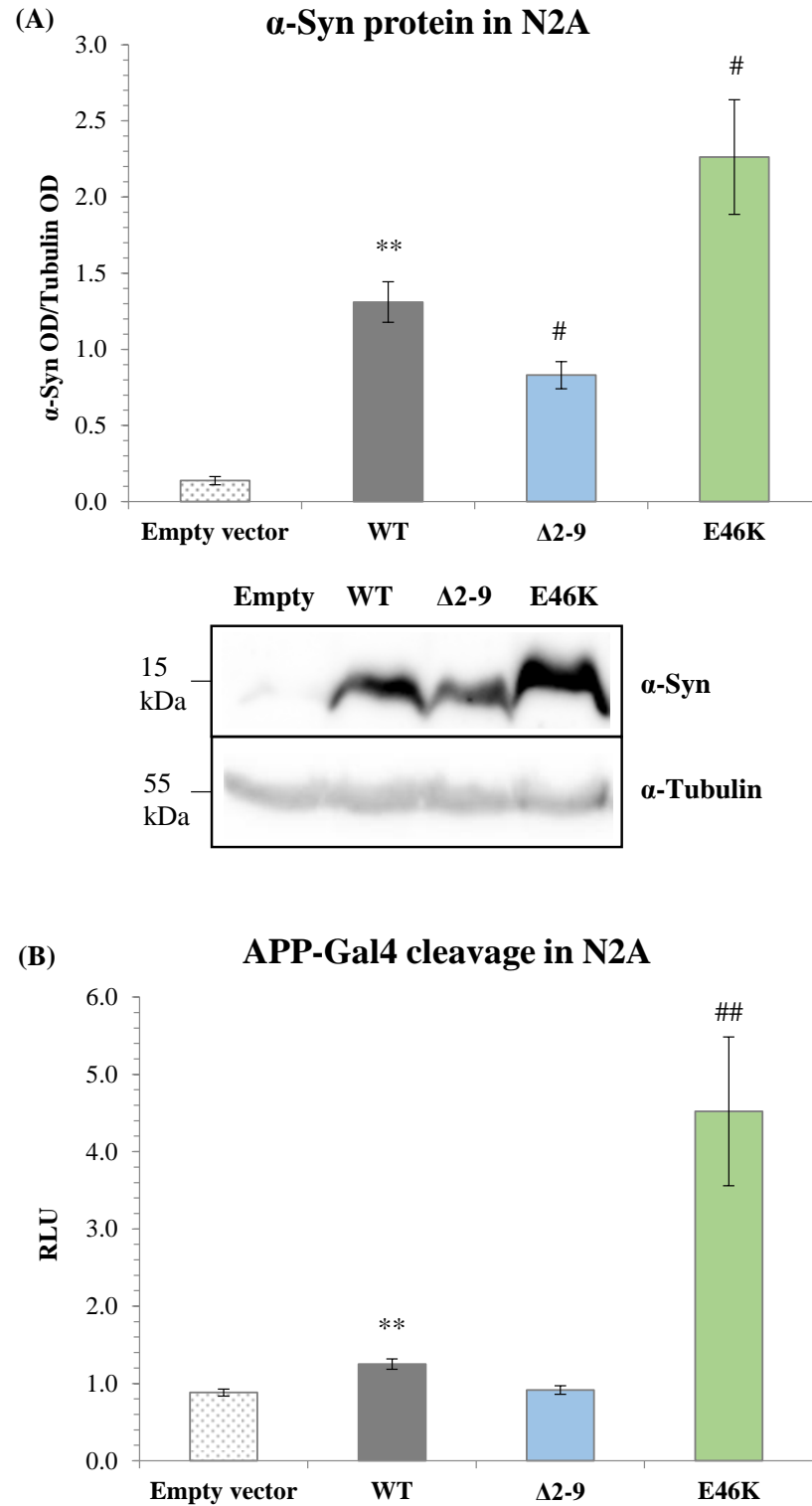


Figure 3.20 Amyloidogenic processing is potentiated in E46K α -syn N2As. (A) Overexpression of α -syn confirmed by western blotting. Whole cell lysates were tested for α -syn and α -tubulin. Mean α -syn OD: tubulin OD for 5 independent experiments. (B) APP-Gal4 reporter activity in mutant α -syn N2As. Mean RLU for 5 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector; # $p < 0.05$, ## $p < 0.01$ relative to WT, calculated by pairwise t-tests with a Holm adjustment.

3.4 Discussion

The results presented in this chapter show that, in a neuronal overexpression model, wildtype α -syn promotes the β -/ γ -cleavage of APP, coinciding with greater extracellular levels of β -amyloid. These results support previous reports that rat neuronal cells treated with α -syn protein upregulate β -amyloid secretion (Kazmierczak et al. 2008; Majd et al. 2013). However, this is the first time that both increased β -cleavage of APP and increased extracellular β -amyloid have been described in an α -syn overexpressing cell model. The effect of wildtype α -syn overexpression is relatively small, but three independent cell lines used to study amyloidogenic processing were in agreement.

Three different, but complementary, techniques were used to study amyloidogenic processing, providing robustness to the conclusions. Each has different strengths and limitations, and measures a different aspect of amyloidogenic processing. The first, an ELISA-style multiplex assay for secreted A β 40 and A β 42, has the advantage of being quantitative and precise. However, the levels of extracellular A β 40 and A β 42 are affected by more factors than β -/ γ -cleavage of APP. Degradation of β -amyloid by proteases such as neprilysin and insulin-degrading enzyme is also important (Wang et al. 2006). Another limitation of the method is that in order to accumulate a measurable quantity of β -amyloid peptides, the conditioned media is collected after 72 hours, which could limit nutrient availability to the cells. The second assay, an APP-Gal4 luciferase reporter for endogenous β -/ γ -cleavage activity at APP, is also quantitative but has the advantage not being confounded by protease activity. Yet measurement is indirect, may alter APP localisation, and is heavily reliant on uniform transfection efficiency. Thirdly, western blotting was performed on the cells for the immediate proteolytic products of β - (C99) and α - (C83) secretase cleavage in the presence of a γ -secretase inhibitor. This is advantageous for being direct and allowing endogenous β -secretase activity to be isolated from γ -secretase activity. However western blotting is not fully quantitative, and the perturbation of γ -secretase activity may affect transcription of the β -secretase protein BACE1 (Tamagno et al. 2008). It is this important to view the results from these three assays collectively. Together they strongly suggest that β -cleavage of APP, and total levels of secreted A β , are specifically elevated by α -syn overexpression.

Interestingly the A β 42:A β 40 ratio was unaltered when α -syn was over-expressed, despite being more highly secreted. A β 42 and A β 40 result from the heterogenous cleavage pattern of γ -secretase, which also creates trace amounts of other A β peptides (Hubin et al. 2014). γ -Secretase preferentially produces A β 40, but saturation of the enzyme with its substrate, C99, leads to longer A β products such as A β 42 (Svedružić et al. 2012). Paradoxically, the A β 42:A β 40 ratio can be increased by γ -secretase inhibition DAPT, due to C99 saturation

(Svedružić et al. 2013). The γ -secretase inhibitor DAPT did increase A β 42:A β 40 in the present study, but all other ratios were constant. One can infer from this that γ -secretase activity is not limiting in α -syn cells.

The experiments focus on a synucleinopathy cell model where α -syn is stably over-expressed in a neuronal cell line. Although this model is fairly ubiquitous in the literature, there is a dearth of literature using the model to study APP processing, which needs to be addressed. Only one paper studied APP in α -syn SH-SY5Ys but focussed on APP expression rather than processing (Jesko et al. 2014). The data in this thesis does not support changes to APP expression when α -syn is over-expressed, or vice versa. Others have approached the question of whether α -syn affects APP by applying recombinant α -syn protein to cells (Kazmierczak et al. 2008; Majd et al. 2013). α -Syn protein addition to cells, whether aggregated or non-aggregated, risks causing only acute toxicity, which is a good model of cell death but a poor model for studying the cell biology of a chronic condition. Inducible expression has a similar issue. For example intracellular calcium levels and calcineurin activity were demonstrated to be exquisitely sensitive to inducible α -syn expression, in a variety of cell models, resulting in dose-dependent cell death (Caraveo et al. 2014). Stable α -syn overexpression avoids acute toxicity, since there is selection for the cells that thrive so cells necessarily adapt to α -syn production. The limitation of this approach is that α -syn overexpression may cause it to saturate physiological sites of action and build up in non-physiological sites (Kahle et al. 2000). Yet reduced α -syn clearance may be a major feature of synucleinopathy disease, leading to pathological accumulation in cells (Stefanis 2012). High intracellular levels of α -syn are known to profoundly alter cell biology, independently of toxic aggregates. α -Syn overexpression may, through its interaction with membranes, impede the secretory pathway and negatively affect the regulation of mitochondrial networks (Su et al. 2010; Wang & Hay 2015; Kamp et al. 2010; Nakamura et al. 2011; Guardia-Laguarta et al. 2014). α -Syn is also proposed to interact with histones and appears to alter histone acetylation (Jin et al. 2011; Kontopoulos et al. 2006; Goers et al. 2003). One study highlights that α -syn expression alters transcription of approximately 600 genes, in wildtype α -syn transgenic mice. Importantly, the gene expression profile is radically changed by aging (Miller et al. 2007). One can thus easily argue that stable α -syn overexpression models have relevance to disease.

The pro-amyloidogenic effect of α -syn that was observed in SH-SY5Ys appears to be specific to neuronal cell types. APP and secretase expression is conserved in non-neuronal cells (Jacobsen & Iverfeldt 2013), yet differences to the regulation of the amyloidogenic pathway have been reported between neuronal and non-neuronal cells (Belyaev et al. 2010; Hong et al. 2012; Jacobsen & Iverfeldt 2013). The difference could potentially arise from

direct competition of α -secretase and β -secretase for APP. This ‘coupling’ of activity is strong in non-neuronal cells, but appears weaker or absent in neuronal cells (‘incomplete coupling’). For example, pharmacological induction of α -secretase activity decreases β -amyloid production in non-neuronal cells (e.g. HEK293, CHO), but does not reduce β -amyloid in SH-SY5Ys and primary neurons (LeBlanc et al. 1998). Inhibition of α -secretase activity increases β -amyloid production in non-neuronal cells (Skovronsky et al. 2000), but does not affect β -amyloid production in primary neurons (Blacker et al. 2002). The APP CTF data in Section 3.3.4 suggests that α -secretase and β -secretase activity are both enhanced in wildtype α -syn SH-SY5Ys, supporting incomplete coupling. APP CTFs were not measured for the HEK293s but one could hypothesise that the α -CTF and β -CTF would exhibit an inverse relationship due to coupling.

Mutant α -syn constructs were tested to shed some light on the structural characteristics of α -syn that may be involved in its effect upon APP processing. Perhaps surprisingly, many different mutations of α -syn acted to *increase* its effect on APP in SH-SY5Ys. N2A lines with mutant α -syn were also tested for APP-Gal4 cleavage, but interpretation of the data was complicated by differences in α -syn protein expression. In SH-SY5Ys, both aggregate-promoting (A30P, E46K, A53T) and aggregate-inhibiting (Δ NAC) mutations potentiated APP-Gal4 cleavage in SH-SY5Ys. The mutation with greatest effect was Δ 2-9, which may alter the subcellular localisation of α -syn and inhibit aggregation (Burré et al. 2012; Wang et al. 2010). It is therefore likely that loss-of-function, rather than gain-of-toxicity, is responsible for the effects of α -syn. Clearly the underlying mechanism is somehow influenced by the structural conformation of the N-terminal domain of α -syn, but there is insufficient information to suggest a mechanism. Interestingly, a phosphorylation-blocking point mutation of S129 in the C-terminal domain appeared to slightly reduce APP-Gal4 cleavage. This result is intriguing given that the S129A mutation has been shown in certain models to delay synuclein-induced neurodegeneration (Chen & Feany 2005; Kragh et al. 2009; Febbraro et al. 2013; Sato et al. 2011). S129 phosphorylation has been shown to mediate the protein-protein interactions of α -syn to proteins such as Rab8a, involved in vesicle trafficking (Yin et al. 2014). When over-expressed, α -syn appears to disturb the function of Rab proteins in vesicle trafficking (Gitler et al. 2008). Perhaps the disruption of vesicle trafficking could be involved in α -syn-induced amyloidogenic processing of APP.

Changes to APP-Gal4 activity in SH-SY5Ys were only partially echoed by the production of β -CTFs and β -amyloid. In Δ NAC and A53T cells there was an increase in APP-Gal4 cleavage but not β -amyloid secretion. A weaker upregulation of β -amyloid than APP processing is logical, since increased amyloidogenic processing of APP is known to enhance

expression of the amyloid degrading enzyme neprilysin (Pardossi-Piquard et al., 2005). The effects of upregulating amyloidogenic processing may therefore be attenuated. However, the effect of E46K on β -amyloid secretion is much higher than one would anticipate based on its APP-Gal4 cleavage. E46K had little effect on APP-Gal4 cleavage but robustly increased β -CTF production and β -amyloid secretion. This highlights the value of using multiple techniques to study a complex phenomenon.

CHAPTER 4: EFFECT OF α -SYN ON THE SECRETASES

4.1 Introduction

4.1.1 Secretases in synucleinopathy disease

The previous chapter revealed changes to secretase-mediated processing of APP in α -syn cell lines. Without increasing APP expression, cells overexpressing α -syn performed more amyloidogenic processing and appeared to secrete more β -amyloid. Increased amyloidogenic processing must result from either elevated β - or γ -secretase activity on APP, or decreased α -secretase activity. The purpose of this study is to identify which secretases have altered activity in α -syn cells, and investigate secretase expression. The wealth of literature on the secretase regulation, summarised briefly in this introduction, means that specific changes to secretase expression can hint at a mechanism by which α -syn acts.

The effect of α -syn upon secretase enzymes has not been previously investigated. Yet potential changes to secretases have been detected in synucleinopathy brains. One study of post-mortem brain tissue noted that PD and DLB brains had elevated BACE1 mRNA levels in the superior frontal gyrus relative to healthy controls, an effect not seen in AD brains (Coulson et al. 2010). In another study, a major component of the γ -secretase complex, presenilin 1 (PS1), was shown to interact with α -syn in amygdala of cognitively normal human brains. DLB amygdala tissue showed elevated interaction of PS1 and α -syn, independently of whether or not amyloid plaques were detected (Winslow et al. 2014). This finding is intriguing, although it remains to be seen whether the interaction has physiological consequences.

4.1.2 Cell regulation of ADAM10 activity

The ‘A Disintegrin And Metalloproteinase’ 10 (ADAM10) is one of several members of the ADAM family of zinc-binding transmembrane proteinases, two of which have been proposed to perform α -secretase activity (Endres & Fahrenholz 2012). Only ADAM10 has been convincingly shown to significantly reduce α -secretase activity when knocked-out in primary neurons (Kuhn et al. 2010) or animal models (Jorissen et al. 2010).

Transcription of ADAM10 can be controlled by two regulatory elements in the ADAM10 promoter that bind retinoid X-receptor (RXR). RXR binds as a heterodimer, with either the retinoic acid receptor (RAR) (Prinzen et al. 2005), or the peroxisome proliferator-activated receptor- α (PPAR α) (Corbett et al. 2015). It has been demonstrated that RXR-RAR dimers, activated by retinoic acid, can increase ADAM10 transcription (Prinzen et al. 2005; Tippmann et al. 2009). Interestingly, upregulation of ADAM10 expression using the synthetic retinoid acitretin results in decreased A β levels, in the cortex of AD mice (Tippmann et al. 2009). Another transcriptional regulator of ADAM10 is a spliced isoform of X-box binding protein 1 (XBP1), generated as a product of the unfolded protein response. ADAM10 mRNA levels increase when ER stress is induced using thapsigargin or tunicamycin. (Reinhardt et al. 2014). XBP-1, introduced by targeted gene therapy, protected mice from dopaminergic neuron death in a neurotoxin-based model of PD (Valdés et al. 2014). ADAM10 transcription is also positively regulated by SRY-related high mobility group box 2 (Sox-2), a transcription factor that controls stem cell fate and adult neurogenesis. Sox-2 levels are reduced in AD brains, and negatively correlate with disease severity (Sarлак et al. 2015).

Translation of ADAM10 can be negatively regulated via binding of a microRNA, miR-144, to the ADAM10 3'UTR. Levels of miR-144 are elevated in AD patients, and appear to be regulated by AP-1/c-Jun (Cheng et al. 2013). ADAM10 undergoes post-translational cleavage of an auto-inhibitory pro-domain (Anders et al. 2001), and then N-terminal glycosylation in the Golgi (Escreveinte et al. 2008). Tight regulation of ADAM10 activity occurs after maturation, through translocation between plasma membrane and Golgi compartments. ADAM10 is most active as an α -secretase at the plasma membrane, although some α -cleavage of APP occurs in the Golgi (Agostinho et al. 2015). In response to receptor-mediated activation of protein kinase C (PKC), synapse-associated protein-97 (SAP97) binds ADAM10 in Golgi outposts and increases its delivery to the post-synaptic membrane (Saraceno et al. 2014). Long term depression (LTD) also stimulates ADAM10 translocation to the plasma membrane, and long term potentiation (LTP) stimuli have the opposite effect, increasing internalisation of ADAM10. LTP activates clathrin-mediated endocytosis of ADAM10, through enhanced association with the clathrin adaptor AP2 (Marcello et al. 2013). AD brains exhibit reduced SAP97 activation by PKC (Saraceno et al. 2014), and increased ADAM10-AP2 association (Marcello et al. 2013).

4.1.3 Cell regulation of BACE1 activity

BACE1 is a type I transmembrane protease, and the β -secretase enzyme responsible for amyloidogenic processing of APP. A homologous enzyme, BACE2, also cleaves APP but in a different site that does not produce β -amyloid peptides (Agostinho et al. 2015). The BACE1

promoter can be regulated by a huge array of transcription factors, including AP1, AP2, CREB, GRE, Sp-1, and NF- κ B (Sambamurti et al. 2004), peroxisome proliferator-activated receptor-gamma (PPAR γ) (Sastre et al. 2006), HIF-1 α (Zhang et al. 2007), and NFAT3 (Mei et al. 2015). Many of the transcription factors are activated by cell stress. For example, NF- κ B and AP1 are activated by oxidative stress, HIF-1 α by hypoxia, and PPAR γ by inflammation (Tamagno et al. 2012). CREB is activated by a component of oxidised LDL, and NFAT3 by increases in intracellular calcium levels (Shi et al. 2013; Mei et al. 2015).

Translational regulation of BACE1 is another key regulatory pathway, as the BACE1 mRNA is translationally repressed by its GC-rich 5' UTR (Lammich et al. 2004). De-repression of BACE1 translation occurs when the translational initiation factor eIF2 α is phosphorylated by eIF2 kinases, under conditions of nutrient deprivation, oxidative stress, or endoplasmic reticulum stress (Devi & Ohno 2014; Mouton-Liger et al. 2012; O'Connor et al. 2008). Phosphorylation of eIF2 α at Ser-51 leads to global translation repression, except for a sub-set of mRNAs, largely stress-response genes, that contain an upstream open-reading frame (Harding et al. 2000). These mRNAs, including BACE1, are translationally upregulated (O'Connor et al. 2008).

BACE1 protein exits the ER with an auto-inhibitory pro-domain, which is cleaved before N-terminal glycosylation. The mature protein is constitutively secreted to the post-synaptic plasma membrane, but is most active as a secretase enzyme at low pH. Therefore the majority of β -cleavage of APP occurs in endosomes. APP and BACE1 are internalised by different routes, but are brought together by sorting between early endosomes (Jiang et al. 2014). The endocytic interaction of APP and BACE1 is limited by proteins that promote trafficking of BACE1 to the TGN, including VPS35 in the retromer complex and GGA1, a member of the Golgi-localized γ -adaptin ear-containing ADP ribosylation factor binding proteins (GGAs) (Wen et al. 2011; Wahle et al. 2005; Jiang et al. 2014). Another GGA protein, GGA3, targets BACE1 to lysosomes for degradation. GGA3 is sensitive to caspase cleavage during apoptosis, and inversely correlates with BACE1 expression in the temporal cortex of AD brains (Tesco et al. 2007).

4.1.4 The γ -secretase complex

γ -Secretase is an aspartic protease complex of at least four proteins that are essential for its activity: presenilin (PS1/2), presenilin enhancer-2 (PEN2), nicastrin, and anterior pharynxdefective-1 (APH-1). The catalytic protein is PS1 or PS2, and is regulated by PEN2. Nicastrin and APH-1 scaffold the complex and nucleate assembly. The majority of mature γ -secretase complexes cycle between the ER and Golgi, but about 5% is transported to the

plasma membrane and endocytosed (Agostinho et al. 2015). Trafficking of γ -secretase to the plasma membrane increases if APP is knocked-down, or if phospholipase D1 is over-expressed. Cholesterol-rich lipid rafts in the TGN and endosomes are particularly enriched with γ -secretase complexes (Vetrivel et al. 2004). Lipids rafts also bind APP and β -secretase, but not α -secretase, and their structural integrity is important for cell β -amyloid production (Ehehalt et al. 2003).

γ -Secretase binds C99, the membrane-bound product of β -secretase cleavage of APP. C99 is cleaved to form a long A β peptide of 48 or 49 residues, which is then further cleaved by γ -secretase 3 amino acids at a time, sequentially producing shorter A β peptides. The most common product is A β 1-40, but detectable levels of A β 1-42 also result, which is more hydrophobic and considered to be more toxic. Interestingly, around 244 familial AD mutations are recorded to affect PS1, PS2, or the region of γ -secretase cleavage in APP (Cruts et al. 2012). These mutations can increase or decrease A β 1-40 production from cells, but it appears that their pathogenicity is closely linked to a reduction in maximal catalytic activity of γ -secretase. For example, L166P mutant PS1 is associated with an age of disease onset of 24, and is estimated to have a maximal activity that is only 28-42% of the wildtype protein (Svedružić et al. 2015). Operating close to its maximal activity means that the γ -secretase active site is saturated with C99 substrate. This appears to result in an increased ratio of A β 1-42: A β 1-40 production, and more release of long A β peptides that may also be toxic, e.g A β 1-43 (Svedružić et al. 2012; Svedružić et al. 2015).

In addition to cleaving the cytoplasmic domain of APP, releasing the APP intracellular domain (AICD), γ -secretase has an important role in Notch processing (De Strooper et al. 1999). Notch processing is performed by γ -secretase and α -secretase, but not β -secretase (De Strooper et al. 1999). In this chapter a luciferase reporter for Notch cleavage will be used as a tool for measuring γ -secretase activity (Cox et al. 2014).

4.2 Aims

Chapter 3 showed that α -syn overexpression in neuronal cell types has a pro-amyloidogenic effect on APP processing. This chapter aims to investigate whether specific changes to the expression and activity of the α -, β -, and γ -secretases underpin the pro-amyloidogenic effect of α -syn. Endogenous γ -secretase activity will be assayed in α -syn SH-SY5Ys, using a luciferase reporter. For the inducible α -secretase ADAM10 and the β -secretase BACE1, a more detailed exploration of transcriptional activity, protein expression and maturation will be performed. Once secretase expression and activity has been characterised in WT α -syn cells, the effects of α -syn mutations will be investigated.

4.3 Results

4.3.1 A post-transcriptional reduction in mature ADAM10 protein within WT α -syn SH-SY5Ys

α -Secretase activity upon APP prevents the formation of β -amyloid by β -secretase cleavage. The extent to which direct substrate competition occurs is not clear, given that mature α - and β -secretases localize to separate membranes, but also co-exist in the TGN. However, in Chapter 3 it was shown that α -secretase inhibition in SH-SY5Ys significantly enhances the signal of a luciferase reporter assay for β -/ γ -secretase activity. Thus it is important to investigate changes to α -secretase expression and activity in α -syn SH-SY5Ys.

ADAM10 is the major α -secretase (Kuhn et al. 2010). Expression of ADAM10 was initially measured by testing its promoter activity. A construct with a fragment of human ADAM10 promoter in control of a luciferase gene was transfected into WT (v1) α -syn SH-SY5Ys. Known regulatory elements for transcription factors RXR and XBP-1 were present in the 2.2 kb promoter fragment (Prinzen et al. 2005; Reinhardt et al. 2014). No significant reduction in promoter activity is evident (Figure 1.1a). ADAM10 protein levels were then measured by western blotting, using whole cell extracts of the WT (v1) α -syn cells (Figure 4.1b). In contrast with the promoter activity, ADAM10 protein levels were significantly decreased in α -syn cells. The change in expression is likely post-transcriptional.

Decreased ADAM10 expression does not necessarily mean that α -secretase activity will be reduced. ADAM10 is produced as an inactive precursor that undergoes maturation in the Golgi, so changes in ADAM10 levels could be counteracted by an opposite change in maturation. To measure the overall ADAM10 activity of the cells, a commercial *in vitro* activity assay was used on whole-cell lysates. The assay specifically tests ADAM10 α -secretase activity with a cleavable fluorometric peptide reporter (Eurogentec), incubated with equal concentrations of cell protein. Fluorescence increased linearly for an hour, and was compared between cell extracts at the 30 minute time-point. ADAM10 catalytic activity was 15% lower in extracts from WT α -syn SH-SY5Ys relative to empty vector cells (Figure 4.1c). This is comparable with the 20% reduction in levels of ADAM10 protein, which may be entirely responsible for the decreased *in vitro* activity. The evidence suggests that there is an overall reduction in mature ADAM10 in α -syn SH-SY5Ys. More evidence would be needed to support a real decrease in α -secretase cleavage of APP, since this is also controlled by sub-cellular localisation.

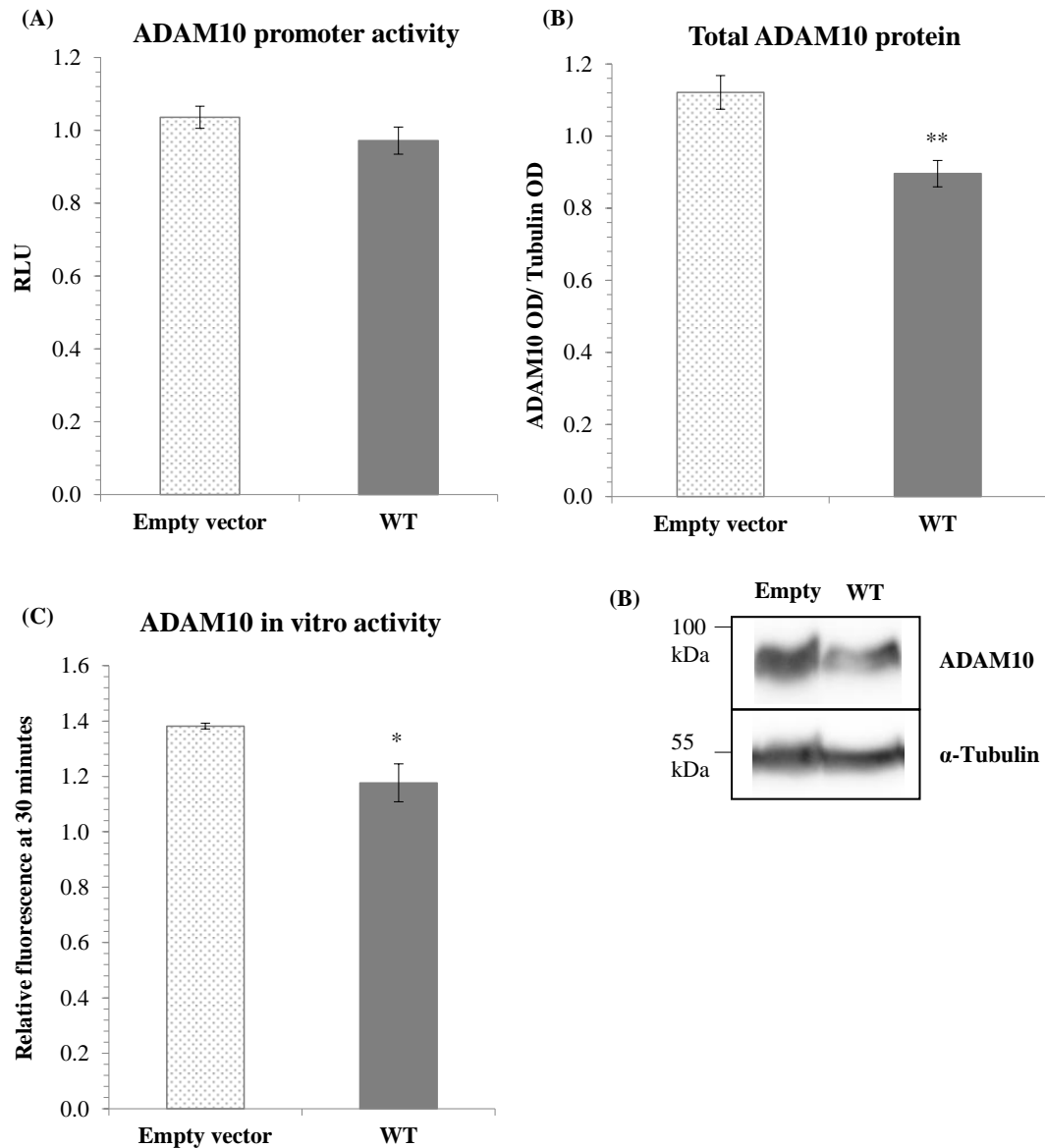


Figure 4.1 In WT α -syn SH-SY5Ys there is a post-transcriptional reduction in levels of active ADAM10. Transcriptional activity of ADAM10 in WT α -syn SH-SY5Ys, measured with a luciferase reporter for a fragment of the human ADAM10 promoter. Mean RLU for 4 independent experiments \pm S.E. **(B)** ADAM10 total protein levels in WT α -syn SH-SY5Ys, measured by western blotting ADAM10 in whole cell lysates. Mean ADAM10 OD: α -tubulin OD for 9 independent experiments \pm S.E. **(C)** *In vitro* ADAM10-mediated catalytic activity from cell extracts of WT α -syn SH-SY5Ys. 50 μ g of total protein from whole cell lysates were incubated with 5-FAM FRET substrate (SensoLyte® 520 ADAM10 Activity Assay Kit, Eurogentec). Mean fluorescence for 4 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

4.3.2 Increased translocation of ADAM10 to the plasma membrane may counteract α -syn-induced changes ADAM10 expression

Levels of active ADAM10 protein were reduced in WT α -syn SH-SY5Ys, but it was unclear whether this necessitated a reduction in its cleavage of APP. Secretase activity is highly compartmentalised, and α -cleavage of APP is thought to mostly occur at the plasma membrane, regulated by receptor-stimulated trafficking (Agostinho et al. 2015). Therefore the cell surface expression of ADAM10 was measured biochemically in WT (v1) α -syn SH-SY5Ys (Figure 4.2). Proteins on the surface of intact cells were biotinylated, the cells lysed, and biotinylated proteins affinity-purified with NeutrAvidin beads. Western blotting was used to compare ADAM10 levels in the purified fraction with input homogenates. A significantly higher proportion of cell surface ADAM10 was detected in WT α -syn SH-SY5Ys compared with empty vector cells. Note that input homogenate ADAM10 levels are not identical, so it is not clear that cell surface expression of ADAM10 is genuinely higher. At the very least, the data suggests that decreased ADAM10 expression in WT α -syn cells does not limit its cell surface availability. One can see that the 26% increase in proportional cell surface expression of ADAM10 is of a similar magnitude to the 20% decrease in total ADAM10 protein levels. It is likely that the two balance out, resulting in similar levels of ADAM10 being localised to the plasma membrane in WT α -syn and empty vector cells.

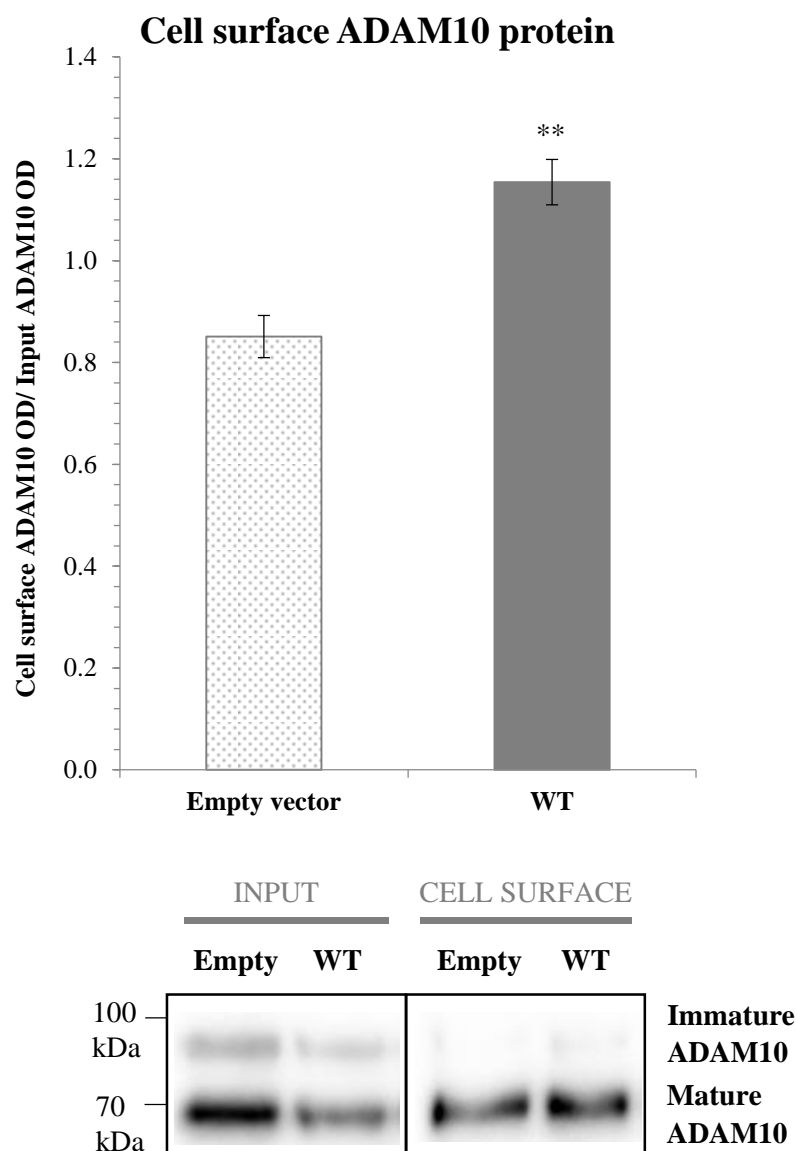


Figure 4.2 Cell surface expression of ADAM10 protein in WT α -syn SH-SY5Ys. The cell surface proteins of intact cells were labelled with Sulfo-NHS-SS-Biotin, then lysed and affinity-purified using NeutrAvidin agarose beads (Cell Surface Protein Isolation Kit, Thermo Scientific). Western blotting for ADAM10 was performed on both purified cell surface protein and the input homogenates. Data shown as the mean ratio of cell surface: total ADAM10 for 4 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

4.3.3 The effect of α -syn on ADAM10 expression is unaltered by selected α -syn mutations

Overexpression of WT α -syn reduced the levels of active ADAM10 protein in SH-SY5Ys. The role of the α -syn N-terminal domain sequence was subsequently investigated, using the mutant α -syn SH-SY5Y lines characterised in Chapter 3. The transcriptional activity of ADAM10 in mutant lines was measured by a luciferase reporter construct, as described previously. Similar to the WT line, human ADAM10 promoter activity was unaltered by any of the disease-associated point mutations (A30P, A53T & E46K; Figure 4.3a). Unexpectedly, the two N-terminal truncations (Δ 2-9 & Δ NAC) caused a significant increase in the apparent promoter activation of ADAM10.

Total levels of ADAM10 protein were also measured by western blotting of whole-cell lysates (Figure 4.3b). Westerns included the two N-terminal truncation mutants (Δ 2-9 & Δ NAC), two disease-associated mutants (E46K & A53T), and two phosphorylation mutants (S129A & S129D). Compared with the WT (v1) α -syn line in adjusted t-tests, none of the mutations have a significant effect on ADAM10 protein levels. Clearly although the N-terminal truncations appeared to upregulate ADAM10 transcription, production of the ADAM10 protein is highly regulated and did not increase.

The *in vitro* catalytic activity of ADAM10 was previously shown to closely follow ADAM10 protein expression in cell extracts. A fluorometric peptide reporter was used that mimics the α -secretase cleavage site of APP (Eurogentec). At the same time as WT (v1) α -syn, cell extracts from the mutant α -syn lines were assayed *in vitro* for ADAM10 catalytic activity (Figure 4.3c). In agreement with the measured levels of ADAM10 protein, there were no significant differences in ADAM10 catalytic activity between the mutant lines and WT α -syn SH-SY5Ys.

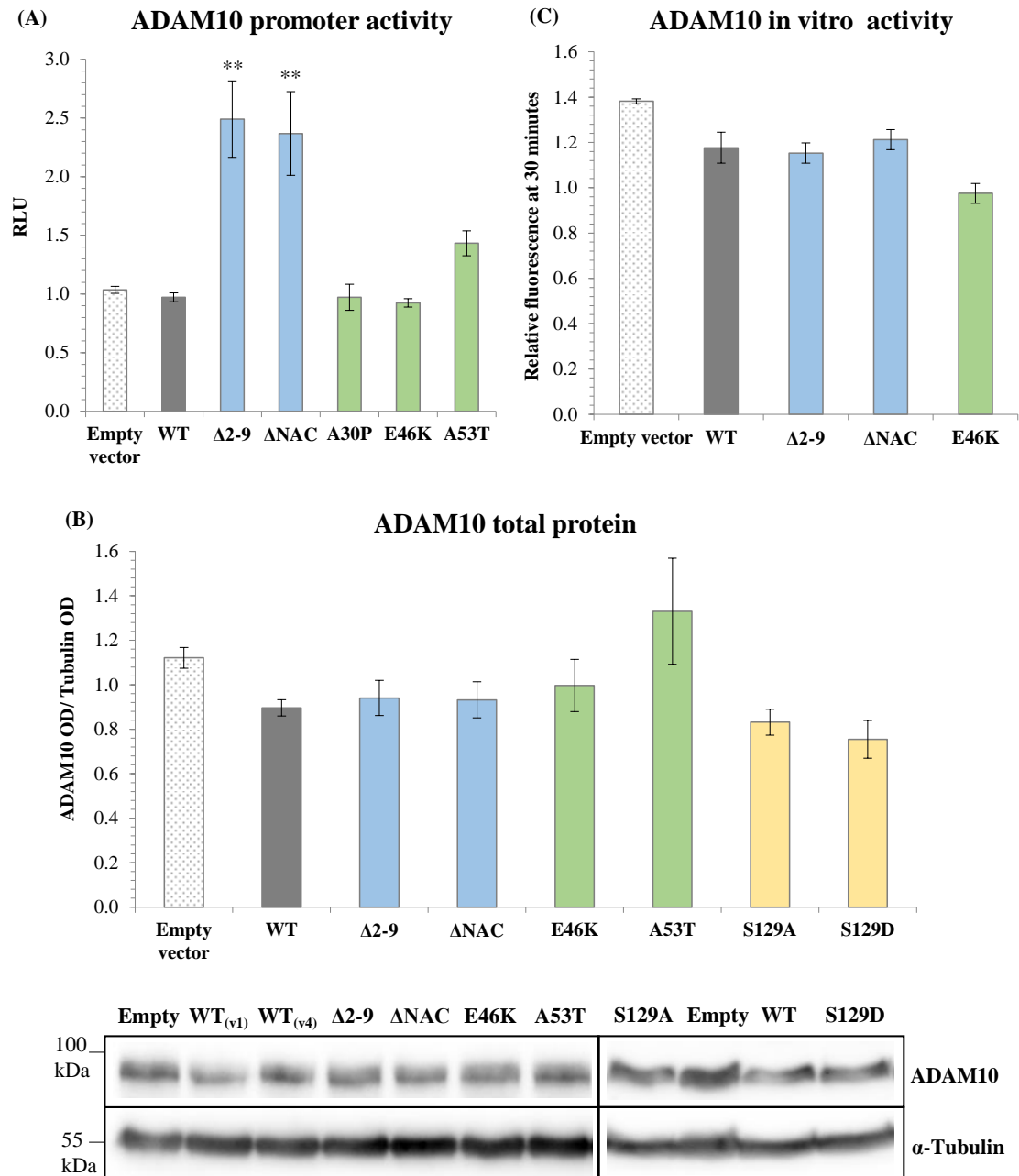


Figure 4.3 α -Syn-mediated reduction of ADAM10 is not affected by mutations. (A) Transcriptional activity of ADAM10 in mutant α -syn SH-SY5Ys, measured with a luciferase reporter for a fragment of the human ADAM10 promoter. Mean RLU for 4-5 independent experiments \pm S.E. (B) ADAM10 total protein levels in mutant α -syn SH-SY5Ys, measured by western blotting ADAM10 in whole cell lysates. Mean ADAM10 OD: α -tubulin OD for 3-7 independent experiments \pm S.E. (C) *In vitro* ADAM10-mediated catalytic activity from cell extracts of mutant α -syn SH-SY5Ys. 50 μ g of total protein from whole cell lysates were incubated with 5-FAM FRET substrate (Sensolyte® 520 ADAM10 Activity Assay Kit, Eurogentec). Mean fluorescence for 3-4 independent experiments \pm S.E. ** $p < 0.01$ relative to wildtype, calculated by pairwise t-tests.

4.3.4 BACE1 expression in WT α -syn SH-SY5Ys is increased

The β -secretase, BACE1, is responsible for amyloidogenic processing of APP. Research has shown that just small increases in BACE1 expression can lead to large increases in β -amyloid production (Li et al. 2006). BACE1 can be regulated at a transcriptional and translational level. Transcriptional activity at the BACE1 promoter was investigated with a luciferase reporter construct, using a fragment of human BACE1 promoter upstream of the firefly luciferase gene (McHugh et al. 2012). All putative transcription factor sites for BACE1 are contained within the promoter fragment (Sambamurti et al. 2004). Promoter activity appeared to be significantly reduced in WT α -syn SH-SY5Ys (Figure 4.4).

BACE1 protein expression was then assessed by western blotting (Figure 4.5a). In contrast to the diminished promoter activity, BACE1 protein levels were significantly elevated in three WT α -syn lines. Interestingly, levels of BACE1 form a similar pattern to α -syn levels in the same cell extracts, highest in 'v3' and lowest in 'v1' (Figure 3.2). Like ADAM10, BACE1 protein is synthesised as an inactive precursor, and matured by pro-domain cleavage and N-terminal glycosylation. Therefore increases in BACE1 expression do not necessarily mean more active BACE1 enzyme. To quantify levels of active BACE1 in cell lysates, a commercial β -secretase activity kit (Eurogentec) was used. Similar to the ADAM10 activity assay, a cleavable fluorometric peptide reporter was mixed with samples of equal protein concentration, and fluorescence recorded at 30 minutes. β -Secretase activity appears higher in the WT (v1) α -syn SH-SY5Ys, but the increase is not statistically significant (Figure 4.5b). Significance may have been affected by high noise in the assay. Increased β -secretase activity in WT α -syn SH-SY5Ys is suggested by data in Chapter 3 that showed elevated production of the β -cleaved C-terminal fragments of APP, 'C99'. Overall, it appears that α -syn overexpression is linked to increased BACE1 protein levels in SH-SY5Ys, and that higher β -cleavage of APP results.

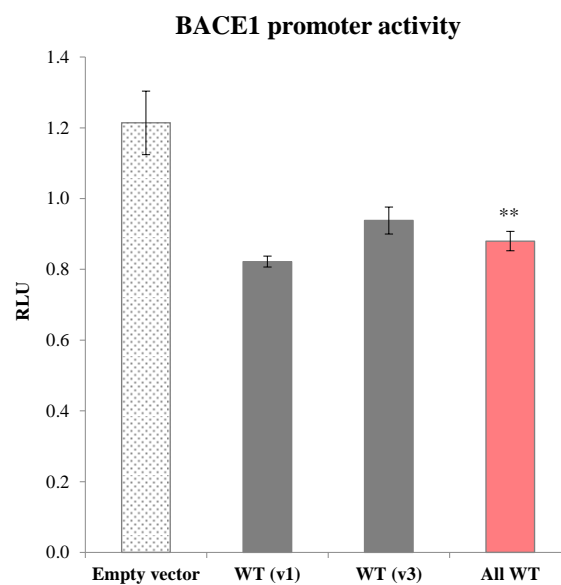


Figure 4.4 BACE1 promoter activity is reduced in α -syn SH-SY5Ys. Transcriptional activity of BACE1 was measured with a luciferase reporter for a fragment of the human BACE1 promoter. Mean RLU for 5 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

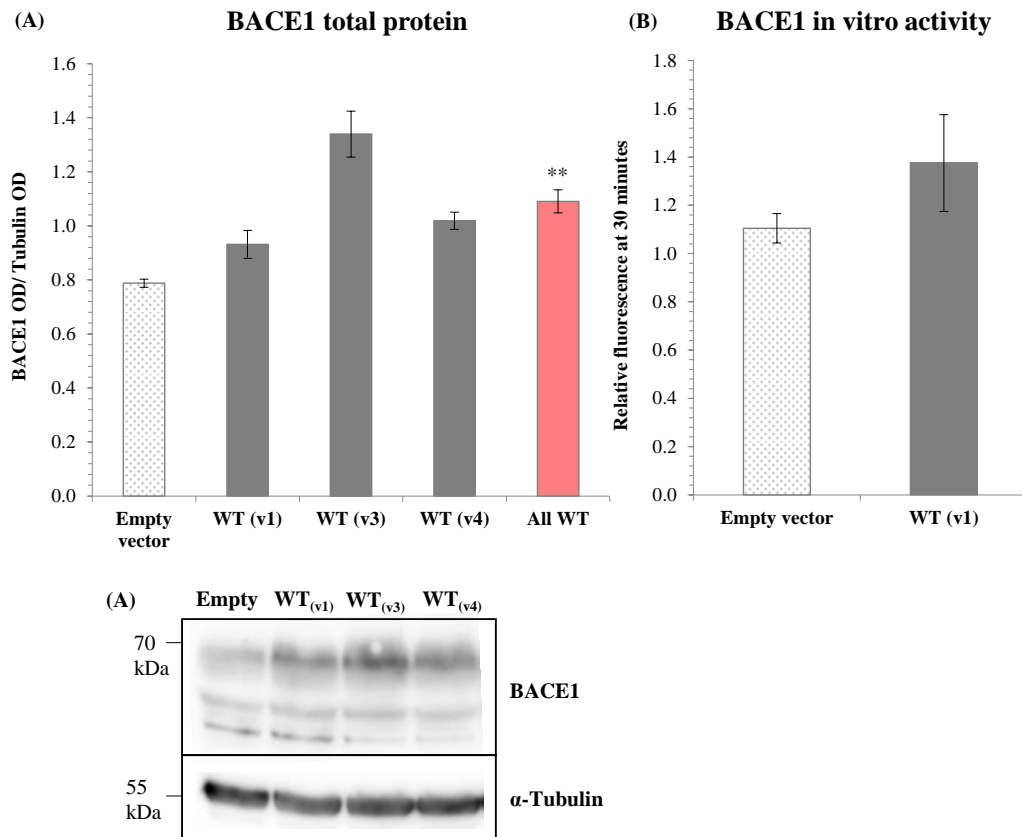


Figure 4.5 BACE1 protein expression is enhanced in α -syn SH-SY5Ys. (A) BACE1 total protein levels in WT α -syn SH-SY5Ys, measured by western blotting BACE1 in whole cell lysates. Mean BACE1 OD: α -tubulin OD for 9 independent experiments \pm S.E. (B) *In vitro* BACE1-mediated catalytic activity from cell extracts of WT α -syn SH-SY5Ys. 50 μ g of total protein from whole cell lysates were incubated with HiLyte™ Fluor 488 FRET substrate (Sensolyte® 520 β -Secretase Assay Kit, Eurogentec), and the fluorescence increase resulting from its cleavage was monitored for 30 minutes. Mean fluorescence for 4 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

4.3.5 The effect of α -syn on BACE1 expression is dose-dependent in SH-SY5Ys

BACE1 protein levels were observed to be highest in the WT α -syn SH-SY5Ys with greatest average α -syn expression, so a dose-dependent effect was hypothesised. In stably-transfected cells, the degree of overexpression cannot be controlled and may vary over time. To clarify whether a correlation between α -syn and BACE1 expression exists, SH-SY5Ys were transiently transfected with different quantities of α -syn plasmid. Both α -syn and BACE1 proteins were measured by western blotting of cell extracts after 48 hours. Transfection of α -syn plasmid results in a strong dose-dependent increase in α -syn protein levels (Figure 4.6a). Correspondingly, BACE1 protein levels appear to mirror the increasing levels of α -syn (Figure 4.6b). The effect is weak however, since only a 1.2- to 1.4-fold change in BACE1 results from a 5.5- to 12-fold change in α -syn. To control for the potential cell stress provoked by overexpression of protein, which alone could account for BACE1 expression, the closely related β -synuclein gene was transfected into SH-SY5Ys. β -Syn does not significantly enhance BACE1 expression, indicating that the effect on BACE1 is specific to α -syn transfection. A small increase in α -syn levels was detected in β -syn cells, but it is likely that there is some cross-reactivity of the α -syn antibody for β -syn. Overall this experiment confirms that BACE1 expression responds to different levels of α -syn overexpression. Furthermore, the result clarifies that a significant change in BACE1 expression occurs within 48 hours, so the effect is not a long-term adaptation by the cell population to selective pressure.

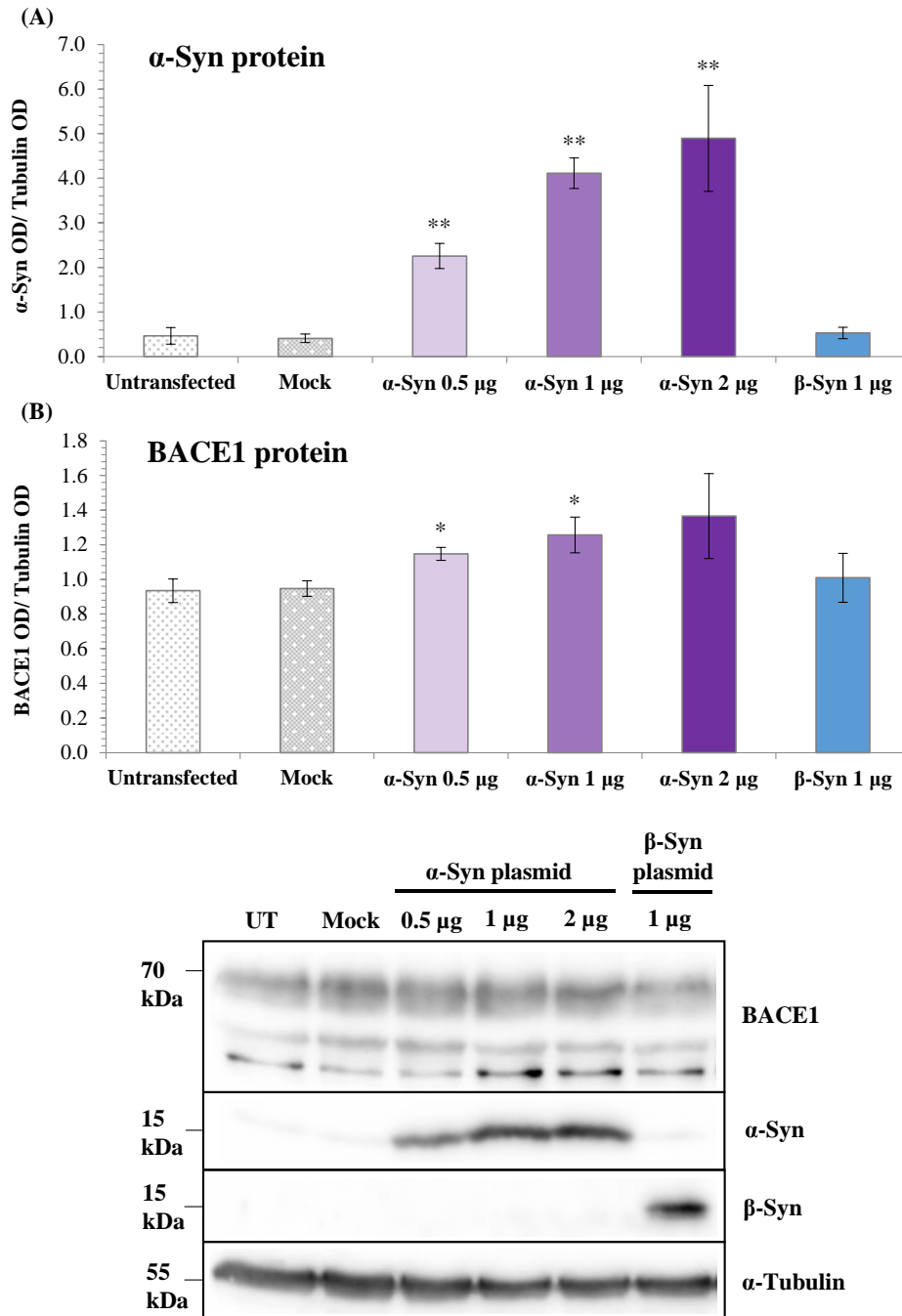


Figure 4.6 Transfection of α -syn dose-dependently increases BACE1 expression. (A) BACE1 and (B) α -syn total protein levels in SH-SY5Ys transiently overexpressing WT α -syn or β -syn. SH-SY5Ys were transfected with 0.5 μ g, 1 μ g, or 2 μ g of pcDNA 3.1(+) - α -syn, or 1 μ g of pcDNA 3.1(+) - β -syn. A mock transfection with no plasmid was also performed to control for transfection-specific effects. Cells were lysed after 48 hours. Whole cell lysates were tested for, α -syn, and α -tubulin by western blotting. Representative blot shown. Mean BACE1/ α -syn OD: α -tubulin OD for 4 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector, calculated by Student's t-test.

4.3.6 BACE1 expression is positively correlated with α -syn expression in transgenic rat striatum

The potential association of α -syn overexpression with BACE1 expression was further investigated *in vivo*. Brain material was obtained from both hemispheres of from rats that were unilaterally injected with α -syn-containing lentivirus into the substantia nigra (Prof. Bernard L. Schneider, EPFL, Switzerland). α -Syn is thought to largely function at presynaptic terminals, and is known to undergo short-range neuron-neuron transmission at synapses (Desplats et al. 2009). Thus the striatum of an infected hemisphere, innervated by dopaminergic neurons from the substantia nigra, is likely to be affected by over-expressed α -syn. Striatal homogenates were tested for BACE1 and α -syn protein levels by western blotting. The striatal samples ipsilateral to the site of vector injection were compared with contralateral samples, acting as internal controls. BACE1 expression was significantly enhanced in the overall population of α -syn-transduced striata relative to controls (Figure 4.7 BACE1 expression is upregulated in α -syn-transduced rat striata).

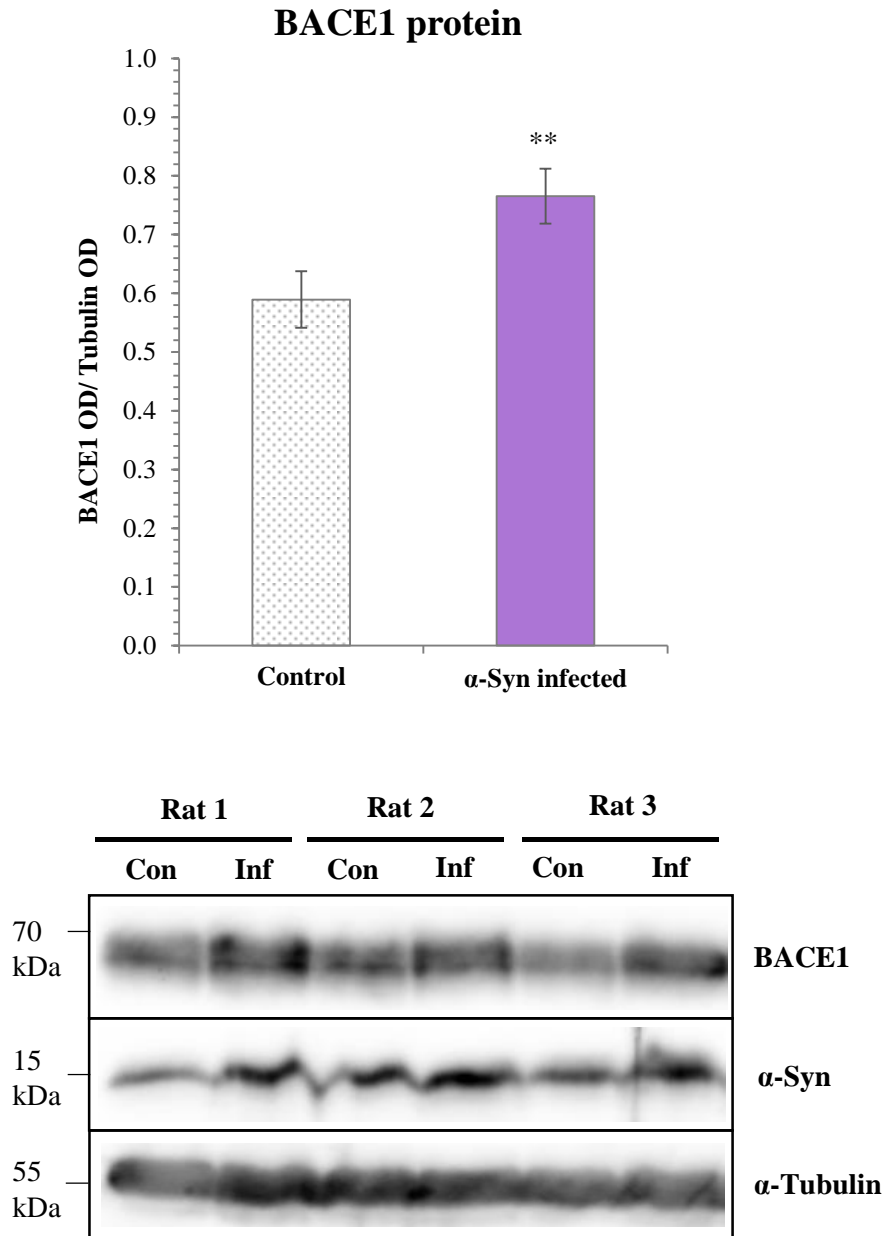


Figure 4.7 BACE1 expression is upregulated in α -syn-transduced rat striata. Striata were obtained from the right and left hemispheres of 8 animals (16 samples in total) that had been unilaterally injected with α -syn lentivirus into the substantia nigra, with thanks to Prof. Bernard L. Schneider (EPFL, Switzerland). Homogenates were tested for BACE1, α -syn, and α -tubulin by western blotting. Representative western blot image of BACE1 expression in three individual rats, of striata ipsilateral ('Inf') and contralateral ('Con') to the injection site. Graph displays mean BACE1 OD: α -tubulin OD for 8 animals, ** $p < 0.01$ relative to control, calculated by Student's t-test.

4.3.7 Specific α -syn mutations can induce BACE1 promoter activation

In Chapter 3, several mutations in the N-terminal domain of α -syn were found to potentiate APP amyloidogenic processing. Earlier in the current chapter these mutations were shown to not affect ADAM10 expression, the hypothetical negative regulator of amyloidogenic processing. Another potential route of influence could be the expression and activity of the β -secretase. Initially, the transcriptional activity of BACE1 was investigated. A luciferase reporter for BACE1 promoter activity was used in several of the mutant α -syn SH-SY5Y lines, as previously described. Similar to the wildtype, activity of the BACE1 promoter reporter was not altered by disease mutants (A30P, E46K, & A53T; Figure 4.8). Interestingly, the Δ 2-9 mutation caused a robust upregulation of BACE1 promoter activity by 2.5-fold. Deletion of residues 71-82 (Δ NAC) also upregulated BACE1 promoter activity, by 1.5-fold. This pattern is familiar from the earlier study of ADAM10 promoter activity. In the case of ADAM10, upregulated promoter activity by Δ 2-9 and Δ NAC did not result in an overall change to ADAM10 protein levels. BACE1 protein levels will be discussed next.

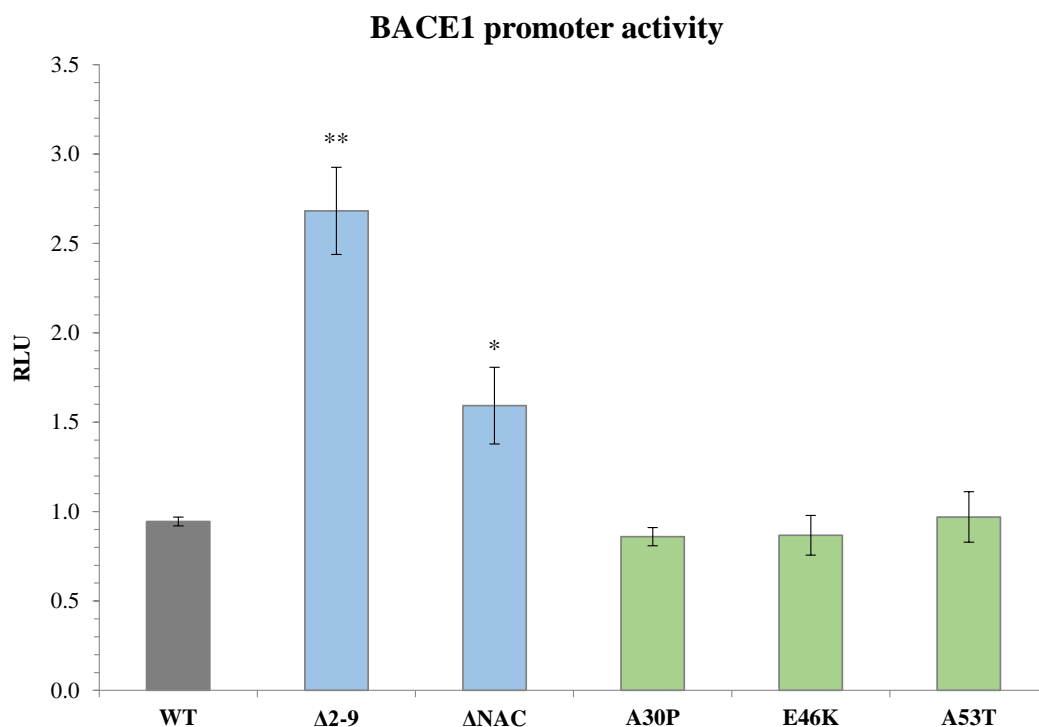


Figure 4.8 Truncation mutants of α -syn increase BACE1 promoter activity. Transcriptional activity of BACE1 was measured with a luciferase reporter for a fragment of the human BACE1 promoter. Mean RLU for 6-7 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to empty vector, calculated by pairwise t-tests with a Holm adjustment.

4.3.8 α -Syn mutations can potentiate its upregulation of BACE1 protein expression

BACE1 protein levels were post-transcriptionally increased by WT α -syn. The result of expressing α -syn mutants upon BACE1 protein levels could aid understanding of how they affect APP amyloidogenic processing. Levels of BACE1 protein were measured in all of the mutant α -syn cell lines for which APP processing was previously characterised (Chapter 3). Mutations are clustered in three groups of relatedness: two N-terminal truncations (Δ 2-9 & Δ NAC), three disease-associated point mutations (A30P, A53T, & E46K), and two C-terminal mutations of the ser-129 phosphorylation site (S129A & S129D). Both N-terminal domain truncations robustly elevated BACE1 protein expression (Figure 4.9), mirroring changes to BACE1 promoter activity. Compared with the WT α -syn line, Δ 2-9 increased BACE1 protein expression by 1.6-fold, and Δ NAC by 1.2-fold, showing marginally weaker potentiation of protein levels than promoter activity. The disease-associated point mutations, A30P and A53T had little effect on BACE1 protein levels. Of the ‘disease’ mutants only E46K significantly enhanced the effect of α -syn upon BACE1. The phosphorylation site mutants S129A and S129D also both appeared to significantly increase BACE1 protein expression. Overall, the changes to BACE1 expression in mutant α -syn lines partially reflect changes to amyloidogenic processing.

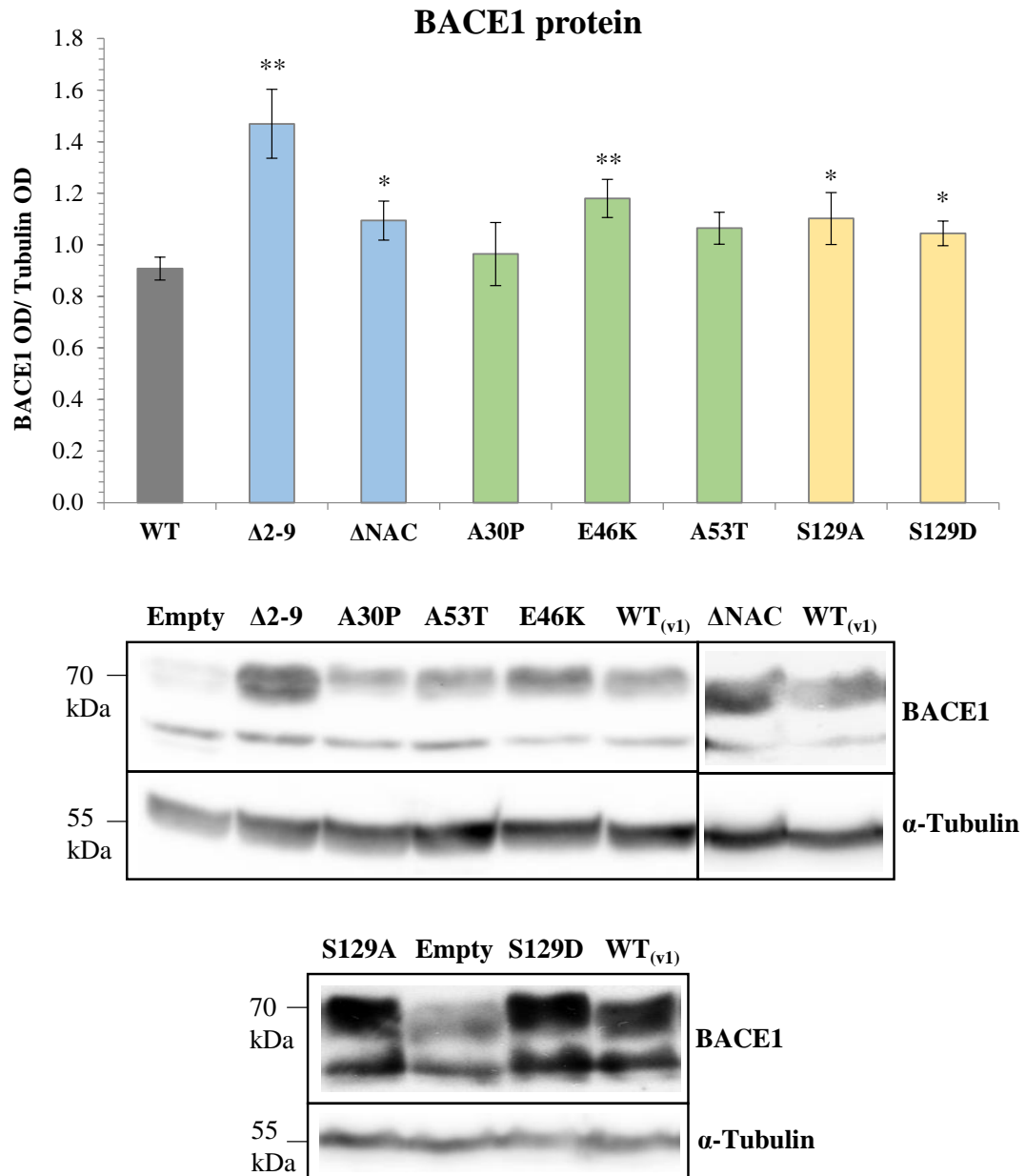


Figure 4.9 BACE1 protein expression is potentiated by several mutations of α -syn. N-terminal domain mutations of α -syn, and C-terminal domain ser-129 mutations of α -syn were over-expressed in SH-SY5Ys. Whole cell lysates were tested for BACE1 and α -tubulin by western blotting. Mean BACE1 OD: α -tubulin OD for 5-12 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to WT (v1), comparisons with WT (v1) were made in three groups (shown in blue, green, and yellow) by pairwise t-tests with a Holm adjustment.

4.3.9 γ -Secretase activity is not affected by WT α -syn overexpression in SH-SY5Ys

Amyloidogenic cleavage of APP and the secretion of β -amyloid are both dependent on γ -secretase activity. Consequently, this is another factor that could potentially contribute to the effect of α -syn expression on amyloidogenic processing. γ -Secretase activity was determined using a Notch-Gal4 luciferase reporter for Notch cleavage (Dr Robert J. Williams, University of Bath). Similar to the APP-Gal4 reporter used in Chapter 3, intracellular γ -secretase cleavage releases the Notch intracellular domain with its fused Gal4 tag, allowing Gal4-mediated transcription of a luciferase construct (pLuc) (Cox et al. 2014). Unlike APP-Gal4, there is no requirement for α/β -secretase activity preceding cleavage of the Notch intracellular domain by γ -secretase. The specificity of the assay for γ -secretase activity was verified using secretase inhibitors (Figure 4.10a). The α -secretase inhibitor TAPI-1 was previously shown to promote APP-Gal4 cleavage, but has no effect on Notch-Gal4 cleavage. γ -Secretase inhibition by DAPT strongly prevents Notch-Gal4 cleavage as one would expect. When transfected into WT α -syn SH-SY5Ys the Notch-Gal4 signal is not significantly altered, which suggests that α -syn has no effect on levels of active γ -secretase (Figure 4.10b).

4.3.10 N-terminal truncated α -syn upregulates γ -secretase activity

γ -Secretase activity is not enhanced in SH-SY5Ys with over-expressed WT α -syn, when measured with a Notch-Gal4 reporter. Levels of active γ -secretase complexes were subsequently measured in several mutant α -syn lines, with the Notch-Gal4 luciferase reporter (Figure 4.11). A striking 4.5-fold increase in Notch-Gal4 cleavage was detected in Δ 2-9 α -syn SH-SY5Ys, compared with the WT (v1) line. The other N-terminal truncation, Δ NAC, did not enhance γ -secretase activity. Disease-associated point mutations A30P, E46K, and A53T also had no significant effect on γ -secretase activity. In the literature, γ -secretase activity has been shown to affect BACE1 transcription (Tamagno et al. 2008), so it is notable that the Δ 2-9 line has both a major increase in γ -secretase activity and significantly enhanced BACE1 transcription.

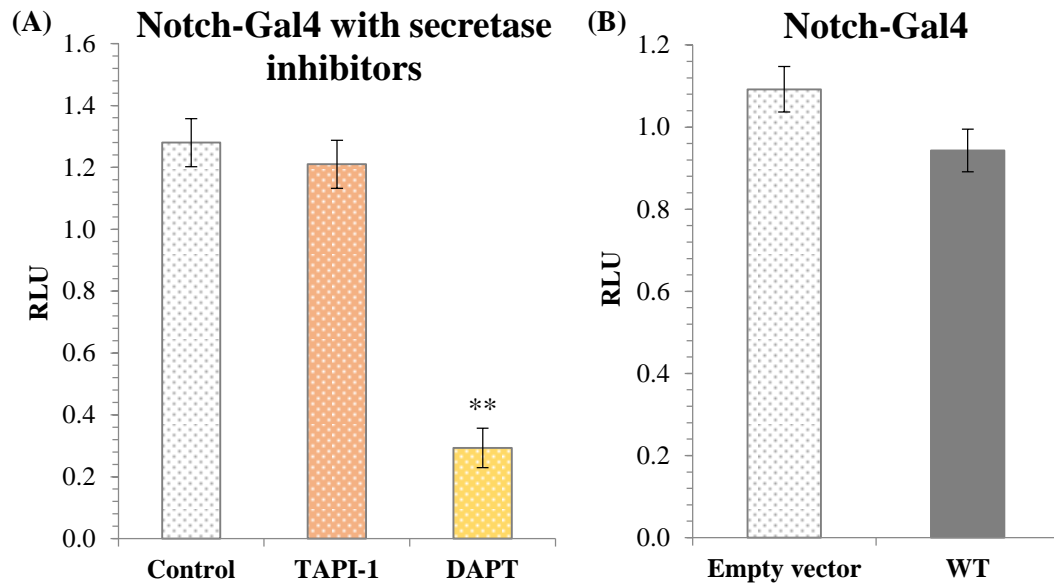


Figure 4.10 Notch-Gal4 reporter for γ -secretase activity in SH-SY5Ys. (A) Notch-Gal4 cleavage is dependent on γ - but not α -secretase. Empty vector SH-SY5Ys were transfected with pTK, pLuc, and Notch-Gal4 constructs. α -Secretase inhibitor TAPI-1 (50 μ M) and γ -secretase inhibitor DAPT (10 μ M) were added 6 hours post-transfection, and incubated 16 hours before lysis. Mean RLU for 3 independent experiments \pm S.E. (B) Notch-Gal4 cleavage in WT (v1) α -syn SH-SY5Ys. Mean RLU for 8 independent experiments \pm S.E. ** $p < 0.01$ relative to empty vector control, calculated by one-way ANOVA with Tukey post-hoc test.

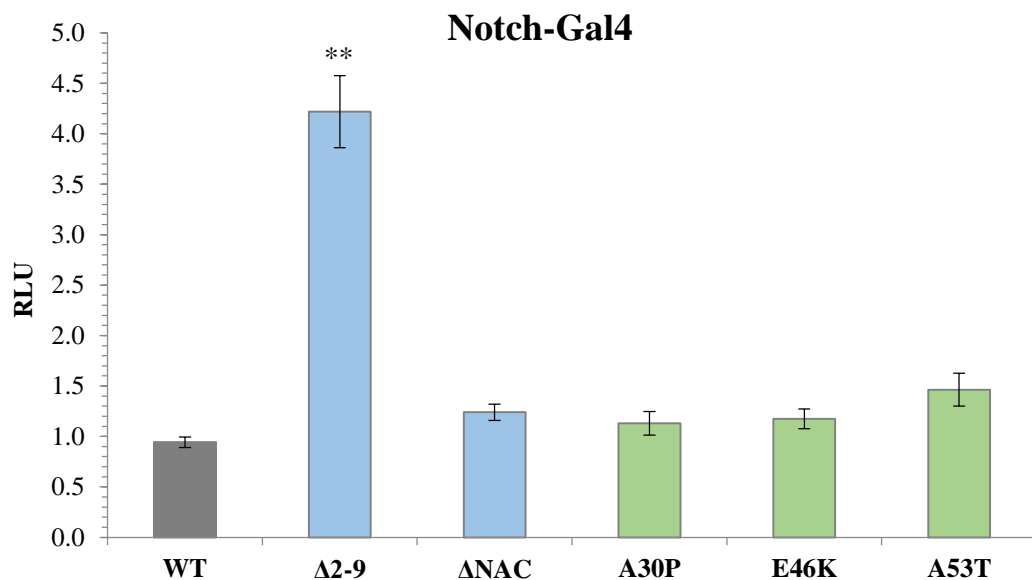


Figure 4.11 γ -Secretase activity appears enhanced by over-expressed $\Delta 2-9$ α -syn. Notch-Gal4 reporter was used to measure γ -secretase activity in mutant α -syn SH-SY5Ys. Cells were transfected with pTK, pLuc, and Notch-Gal4 constructs. Mean RLU for 5-8 independent experiments \pm S.E. ** $p < 0.01$ relative to WT (v1), calculated by pairwise t-tests with a Holm adjustment.

4.4 Discussion

The previous chapter observed that the secretase-mediated processing of APP becomes more amyloidogenic when α -syn is stably over-expressed in neuronal cell lines. Enhanced production of toxic β -amyloid peptides was also detected from α -syn SH-SY5Ys. A complex balance between amyloidogenic and non-amyloidogenic pathways of APP processing exists, involving the expression, activity, and subcellular localisation of three secretase enzymes. This current chapter aimed to discover any major changes to α -, β -, and γ -secretase activity and expression that may underlie the effects of α -syn on APP processing. It was hypothesised that wildtype α -syn may increase β -secretase and/or γ -secretase activity, or reduce α -secretase activity. Expression of WT α -syn in SH-SY5Y cells did not seem to affect γ -secretase activity, so no further investigation was made into γ -secretase. Yet changes to both the α -secretase ADAM10 and the β -secretase BACE1 were revealed.

WT α -syn reduced ADAM10 protein levels. Since promoter activity was not reduced, the effect must be post-transcriptional and not involve transcriptional regulation by RXR complexes or XBP-1 (Prinzen et al. 2005; Reinhardt et al. 2014). Potentially, translational repression could cause the reduction in ADAM10 protein, and this is known to be performed by at least one micro-RNA responsive to cell stress (Cheng et al. 2013). However the increased degradation of ADAM10 protein cannot be ruled out without further investigation. Despite the clear reduction in total levels of active ADAM10 enzyme, confirmed by *in vitro* activity assays, it is not clear whether a physiological reduction in α -secretase activity results. α -Secretase cleavage of APP is thought to largely occur at the plasma membrane, and ADAM10 trafficking to the cell surface is highly regulated (Agostinho et al. 2015). Cell surface levels of ADAM10 were actually found to be increased, as a ratio of the total ADAM10 protein in WT α -syn SH-SY5Ys. Since total ADAM10 is reduced, one cannot conclude that WT α -syn cells have significantly higher cell surface expression than empty vector cells. Yet at the very least this indicates that the overall reduction in ADAM10 protein expression is not reflected in cell surface levels of ADAM10. Further experiments are needed to clarify the role of ADAM10 in α -syn-regulated APP processing.

BACE1 protein levels are increased by WT α -syn overexpression, and it is likely that this increases the pool of catalytically active BACE1. Results of *in vitro* activity assays failed to confirm this, due to high noise. However, indirect evidence of high β -secretase activity in the WT α -syn SH-SY5Ys was previously shown in Chapter 3, in the elevated production of C99 APP. C99 C-terminal fragments of APP are only produced by β -secretase cleavage, and the presence of a γ -secretase inhibitor prevented further metabolism. Paradoxically, BACE1 promoter activity was reduced in WT α -syn SH-SY5Ys, at odds with the increased BACE1

protein levels. This is not without precedent in the literature; overexpression of the prion protein PrP in SH-SY5Ys was previously shown to both reduce human BACE1 promoter activity (same construct) and increase BACE1 protein levels (McHugh et al. 2012). Additionally, the translational upregulation of BACE1 in response to nutrient deprivation corresponds with a drop in BACE1 mRNA levels (O'Connor et al. 2008). It is possible that the drop in BACE1 transcription is a negative feedback response to increased BACE1 translation/stability. NF- κ B is a likely candidate, reported to cause both A β -induced transcriptional repression of BACE1, and A β -induced transcriptional activation of BACE1. The latter is observed when A β production is pathologically high, and the former under physiological conditions (Chami et al. 2012). Regardless, the effect of α -syn on BACE1 protein appears to be post-transcriptional in origin. Likely mechanisms include the eIF2 α -dependent translation of BACE1 mRNA, and the degradation of BACE1 protein by autophagic and proteasomal pathways. Both of these regulatory pathways for BACE1 expression will be investigated in the next chapter.

BACE1 protein expression was further investigated, by examining a range of α -syn expression levels in SH-SY5Ys and rat striatum samples. Transient transfection of SH-SY5Ys with α -syn revealed a positive trend in BACE1 protein levels, with increasing α -syn dose. In transiently transfected SH-SY5Ys it was noticeable that a large 5-fold increase in α -syn levels caused only a 20% increase in BACE1. The weakness of the effect of α -syn on BACE1 raises the question of whether it is physiological. Samples of rat brain striatum allowed the relationship between physiological levels of α -syn and BACE1 to be probed. The individual animals had a range of basal α -syn expression levels in the striatum of one hemisphere, which were slightly increased in the striatum of the other hemisphere where the neighbouring substantia nigra was transduced with α -syn lentivirus. Importantly, there was a significant correlation between BACE1 and α -syn expression across the individual animals in all samples, regardless of whether or not α -syn levels were artificially induced. The correlation of BACE1 expression with physiological levels of α -syn expression, *in vivo*, gives credibility to data obtained from α -syn overexpressing cell models.

Mutant α -syn SH-SY5Y lines allowed the role of α -syn N-terminal sequences to be explored, in terms of changes to ADAM10 and BACE1 expression. Deletion of residues 2-9 or 71-82 were shown in Chapter 3 to promote amyloidogenic processing, relative to the wildtype. Consistent with this, ADAM10 expression was unaffected, BACE1 transcription and protein expression was increased, and γ -secretase activity also enhanced in Δ 2-9 α -syn cells, and to a limited extent Δ 71-82 cells. The transcriptional changes are interesting given that α -syn has been proposed to regulate gene expression. α -Syn has been shown to reduce the

histone acetyltransferase activity of p300 (Jin et al. 2011), which is a co-activator of the BACE1 and PS1 promoters (Lu et al. 2014). PS1 is the major catalytic component of γ -secretase, so a loss-of-function of α -syn could hypothetically increase BACE1 transcription and γ -secretase activity, via increased p300 activity. Besides the transcriptional changes seen in $\Delta 2-9$ cells, post-transcriptional increases in BACE1 expression are also seen in the mutant lines E46K, S129A, and S129D. The disease-associated point mutations did not affect ADAM10 expression or γ -secretase activity, so elevated BACE1 protein levels could be responsible for their increased amyloidogenic processing of APP. However, BACE1 protein levels do not perfectly match the changes in amyloidogenic processing measured by APP-Gal4. A30P and A53T significantly increased APP-Gal4 cleavage, but not BACE1 protein expression. Furthermore, S129A and S129D had opposing effects on APP-Gal4 cleavage, but the same effects on BACE1 protein expression. Potentially, these mutants may additionally alter the sub-cellular localisation of APP with β -/ γ -secretase, which was not studied.

Further investigation is clearly needed to pinpoint the effects of mutating α -syn upon APP processing. As yet, one can only conclude that truncations of the α -syn N-terminus activate APP amyloidogenic processing through a distinct mechanism compared with wild-type α -syn. In contrast, the point mutations largely have the same effects as wild-type α -syn. Both mechanisms appear to converge on BACE1, increasing BACE1 protein levels. Although not perfect consistent, BACE1 protein expression most closely follows APP-Gal4 cleavage, out of the assays used for secretase expression and activity. Thus the upregulation of BACE1 expression by α -syn is worth further investigating, to probe the underlying mechanisms.

CHAPTER 5: POTENTIAL MECHANISMS UNDERLYING THE EFFECT OF α -SYN ON BACE1

5.1 Introduction

5.1.1 Narrowing the focus onto BACE1 expression

In Chapter 3, increased amyloidogenic processing of APP was demonstrated in α -syn overexpressing cells. A number of factors can affect amyloidogenic processing of APP, including increased β -secretase (BACE1) and γ -secretase activity, or decreased α -secretase (ADAM10) activity. Furthermore, increased localisation of APP with lipid rafts and BACE1-positive endosomes can increase amyloidogenic processing. Chapter 4 showed that BACE1 protein levels appear to follow the same pattern as levels of amyloidogenic APP processing, and that BACE1 expression correlates with α -syn expression. γ -Secretase activity was not enhanced, and expression of mature surface-localised ADAM10 was not reduced. The localisation of APP with BACE1 was not studied, and if altered could have a major effect on amyloidogenic processing. However, BACE1 protein levels appear to be a convenient and reproducible “readout” for the effects of α -syn on the amyloidogenic pathway. The purpose of the current chapter is to explore the mechanisms by which α -syn may promote β -secretase-mediated processing of APP, using BACE1 expression to test their likely involvement.

A comprehensive literature search was performed to determine known regulators of BACE1 protein levels, and the cell processes that activate these regulators. The major cell processes that control BACE1 protein levels are largely stress-induced, and can be grouped into four themes: protein degradation, intracellular calcium signalling, oxidative stress, and endoplasmic reticulum (ER) stress. The following literature review will outline the four themes of BACE1 regulation, and additionally the literature supporting a role for α -syn in each.

5.1.2 Proteasomal and lysosomal degradation pathways

5.1.2.1 BACE1 is degraded primarily, but not exclusively, by the lysosome

BACE1 is targeted to plasma membranes by the secretory pathway, yet its activity as a β -secretase is greatest in the acidic environment of endosomes. A dileucine motif directs BACE1 to be sorted to endosomes, where it may then be recycled back to the plasma membrane or degraded by the lysosome (Koh et al. 2005). Lysosomal degradation additionally requires ubiquitination of the dileucine motif, which recruits GGA3 (Golgi-Associated, Gamma Adaptin Ear Containing, ARF Binding Protein 3) for lysosomal targeting of BACE1 (Kang et al. 2012). GGA3 levels are depleted in AD brain samples, most likely due to cleavage by caspase-3 (Tesco et al. 2007). BACE1 is relatively stable with a half-life of 12-16 hours, so recycling may occur a number of times before degradation (Huse et al. 2000). Lysosomal degradation of BACE1 is clearly the main route of clearance, yet BACE1 has also been reported to accumulate in the presence of proteasomal inhibitors (Qing et al. 2004). This result has not been corroborated independently (Koh et al. 2005), but most likely proteasomal inhibition is adequately compensated for by induction of autophagy (Shen et al. 2013; Ding et al. 2003), which would have a greater effect on BACE1 protein levels. The clearest evidence for proteasomal degradation of BACE1 is recent work on the E3 ubiquitin ligase CHIP (C-terminus of Hsc70 Interacting Protein). CHIP overexpression was found to decrease BACE1 protein levels, and could be prevented by proteasome inhibition (Singh & Pati 2015).

5.1.2.2 α -Syn impairs the proteasome and is linked to defective macroautophagy

α -Syn may indirectly impact upon BACE1 levels through impairment of protein degradation pathways. Proteasome block by soluble oligomers of α -syn has been demonstrated *in vitro*, using both PC12 cell-derived mutant protein (Emmanouilidou, Stefanis, et al. 2010) and WT recombinant oligomers (Snyder et al. 2003; Lindersson et al. 2004). In the context of stably-overexpressing cell lines, WT α -syn has little effect on the proteasome, whereas aggregate-promoting mutations A53T and A30P strongly impair proteasomal activity (Stefanis et al. 2001; Tanaka et al. 2001). Proteasome inhibition may thus be an aggregate-specific phenomenon. More importantly in the case of BACE1, over-expressed α -syn is also hypothesised to impair the autophagy-lysosome pathway (Perrett et al. 2015). A number of α -syn transgenic cells and animal models have been found to accumulate autophagosomes, but the interpretation of various studies is contradictory, citing either defective autophagic flux or increased autophagosome formation. The subject is reviewed excellently in (Xilouri et al. 2016). Convincing evidence of defective autophagy was found in Lewy body dementia brains (Crews et al. 2010). Neurons with particularly high α -syn levels had elevated mTOR, a negative

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regulator of autophagy. α -Syn transgenic mice additionally exhibited higher mTOR levels, and pharmacological inhibition of mTOR led to a redistribution of α -syn, and ameliorated dendritic pathology (Crews et al. 2010). The mechanism for which α -syn affects autophagy is not clear. Insoluble α -syn aggregates are resistant to degradation and impair autophagosome clearance in cells (Tanik et al. 2013), but it is likely that unaggregated α -syn affects the early formation of autophagosomes (Xilouri et al. 2016). One proposed mechanism is through impaired vesicle fusion events in the cell (Gitler et al. 2008; Perrett et al. 2015). A number of α -syn-induced vesicle trafficking deficits in PD models have shown to be rescued by overexpression of Rab GTPases (Cooper et al. 2006; Breda et al. 2015), including Rab1a in the case of autophagy (Winslow et al. 2010). In transgenic mice, A30P α -syn co-immunoprecipitates with multiple Rab GTPases, suggesting a functional connection (Dalfó et al. 2004).

5.1.3 Dysregulated intracellular calcium signalling

5.1.3.1 BACE1 transcription is activated by high intracellular concentrations of calcium

Intracellular calcium is one potential cell mediator between α -syn and BACE1. The BACE1 promoter appears to be activated by calcium signalling. Increased intracellular calcium activates calcineurin, which dephosphorylates NFAT (Nuclear Factor of Activated T-cells). The neuron-specific NFAT1/3 isoforms, and the astrocyte-specific NFAT4 isoform, have all been shown to bind the BACE1 promoter and increase BACE1 transcription (Mei et al. 2015; Jin et al. 2012; Cho et al. 2008). Calcium also activates calpain proteases. Overexpression of m-calpain increases BACE1 expression in cell lines, whereas calpain inhibition reduces BACE1 expression and β -amyloid deposition in a transgenic mouse model of AD (Liang et al. 2010). The effect of calpain on BACE1 may be due to increased formation of p25, a product of calpain processing of p35 (Liang et al. 2010). p25/cdk5 complexes activate STAT3, a transcriptional activator of BACE1 (Wen et al. 2008).

5.1.3.2 α -Syn may have a physiological connection to calcium signalling

The physiological role of α -syn in the cell has not been fully defined. It has been suggested that α -syn may regulate intracellular calcium signalling, based on two observations. Firstly, the calcium-activated protein calmodulin (CaM) binds and co-immunoprecipitates with α -syn. This interaction is enhanced by α -syn overexpression in SK-N-SH cells and transgenic mouse brain (Yang et al. 2013). Detailed NMR structural analysis shows that the acetylated N-terminus of α -syn binds directly to calcium-bound CaM, and that this may act as a switch releasing α -syn from membrane-binding (Gruschus et al. 2013; Lee et al. 2002). The purpose of the interaction with CaM is not clear, but Yang et al. (2013) suggest that the complex

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activates Src kinase, which leads to phosphorylation and inhibition of protein phosphatase 2A (Yang et al. 2013). Secondly, α -syn may regulate calcium release from the ER. Cali et al. (2012) showed that α -syn overexpression in cell lines enhances the mitochondrial uptake of calcium, during agonist-stimulated release of ER calcium stores. This appears to be due to increased tethering of the ER and mitochondrial membranes, which occurs in WT but not C-terminal truncated α -syn cells (Cali et al. 2012).

5.1.3.3 α -Syn overexpression disturbs intracellular calcium homeostasis

α -Synucleinopathy models exhibit abnormal calcium homeostasis. The application of recombinant soluble oligomers of α -syn to cells has been repeatedly shown to create cytosolic calcium spikes and activate calcineurin (Danzer et al. 2007; Schmidt et al. 2012; Martin et al. 2012). Stable overexpression of α -syn in SH-SY5Ys results in greater calcium entry after plasma membrane depolarisation. This result was obtained independently by two groups, but they did not agree on whether basal cytosolic calcium levels were enhanced (Hettiarachchi et al. 2009; Furukawa et al. 2006). Yeast studies have shown that α -syn-induced calcium spikes originate from the release of intracellular calcium stores (Caraveo et al. 2014; Büttner et al. 2013). Caraveo et al. generated yeast strains expressing different inducible levels of α -syn. Upon induction, intracellular calcium levels increased dose-dependently with the level of α -syn expression, and were prolonged in the most toxic strain. Furthermore, the importance of calcium-induced calcineurin activation was demonstrated in primary rat neurons. Moderate levels of the calcineurin inhibitor FK506 rescued rat neurons from the toxicity induced by virally transduced α -syn (Caraveo et al. 2014). Calcineurin activation is relevant to BACE1 transcriptional regulation, as mentioned previously, due to the downstream activation of transcription factor NFAT. In α -syn transgenic mice, the increased activation of NFAT3 and NFAT4 was confirmed by their greater nuclear staining. Increased NFAT4 nuclear staining was also detected in human PD and DLB brain sections, particularly in areas associated with α -syn pathology (Caraveo et al. 2014).

5.1.4 Oxidative stress

5.1.4.1 The BACE1 promoter is activated by oxidative stress

It has long been known that BACE1 expression follows β -amyloid levels, implying the existence of a positive feedback loop. A β 42 is likely to mediate positive feedback. Disease-associated mutations of presenilin-1 (PS1) that increase the formation of A β 42 species have been shown to increase BACE1 expression (Giliberto et al. 2009). Oxidative stress also increases PS1 and γ -secretase activity, which appears to promote BACE1 expression in a

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mechanism involving β -amyloid. Crucially, oxidative stimuli do not enhance BACE1 expression in PS1 KO mouse embryonic fibroblasts, unless they are transfected with PS1 (Tamagno et al. 2008; Jo et al. 2010). The γ -secretase inhibitor DAPT also reduces BACE1 expression in oxidative stress-stimulated SH-SY5Ys and unstimulated AD model mice (Jo et al. 2010). The effect of oxidative stress on BACE1 may be partly mediated by transcriptional activation of the BACE1 promoter by NF- κ B or AP-1. Pharmacological inhibition of NF- κ B abrogates A β -induced or 27-hydroxycholesterol-induced BACE1 transcription (Buggia-Prevot et al. 2008; Marwarha et al. 2013). Furthermore, a polyphenol inhibitor of BACE1 transcription has been found to work by disrupting the p65 complex of NF- κ B (Zheng et al. 2015). However, Tamagno et al. (2008) attribute oxidative stress-induced BACE1 expression to the c-jun N-terminal kinase (JNK) pathway, and activation of the transcription factor AP-1. Use of a JNK-1 inhibitor peptide, or JNK KO cell model prevents induction of BACE1 mRNA by oxidative stress (Tamagno et al. 2008).

5.1.4.2 Oxidative stress induces de-repression of BACE1 translation

Promoter activation of BACE1 by oxidative stress cannot be the full story, given that BACE1 is normally translationally repressed by a CG-rich 5'-UTR (Lammich et al. 2004). Phosphorylation of eIF2 α by eIF2 kinases during conditions of cell stress allows improved BACE1 translation (O'Connor et al. 2008). The eIF2 kinase PKR (protein kinase R) controls cell survival in response to a variety of signals, including oxidative stress, calcium stress, and ER stress, as reviewed in (Marchal et al. 2014). PKR activation by virus dsRNA has been shown to lead to β -amyloid accumulation (ILL-Raga et al. 2011), and PKR genetic silencing in mice prevents the induction of BACE1 expression by oxidative stress or neuro-inflammation (Mouton-Liger et al. 2015; Carret-Rebillat et al. 2015). Hydrogen peroxide-stimulated SH-SY5Ys exhibit phosphorylation of eIF2 α and PKR activation, in addition to BACE1 protein upregulation. In this scenario, pharmacological inhibition of PKR abrogates BACE1 expression, whereas inhibition of eIF2 α phosphatase activity potentiates BACE1 expression (Mouton-Liger et al. 2012).

There is far from a consensus on the relative contribution of transcriptional or translational pathways, upregulating BACE1 in response to stress. Two studies challenge the importance of eIF2 α phosphorylation in regulating BACE1 expression. In the first study, the genetic reduction of eIF2 α phosphorylation was achieved in primary neurons and AD model mice by two approaches: overexpression of GADD34, which targets the phosphatase to eIF2 α , or knock-in of non-phosphorylatable S51A eIF2 α . Yet low eIF2 α phosphorylation failed to prevent the induction of BACE1 protein levels in response to A β oligomers (Sadleir et al. 2014). The study convincingly demonstrates that BACE1 expression is not entirely dependent

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upon translational de-repression by eIF2 α . However, it does not prove that eIF2 α does not have a significant role in BACE1 expression, since silencing eIF2 α would likely upregulate other stress pathways, including those that regulate BACE1 transcription. The second independent study, used primary neurons infected with adenovirus containing tagged BACE1. In response to A β oligomers, the adenovirus BACE1 protein increased. Interestingly, this exogenous BACE1 did not have a 5'-UTR for eIF2 α to control, or an ordinary promoter, therefore the authors concluded that the effect was post-translational (Mamada et al. 2015). Oxidative stress-induced changes to BACE1 subcellular localisation have been observed previously, and may provide another layer of regulation to β -secretase activity (Tan et al. 2013). Clearly the literature detailing the control of BACE1 expression and activity in neurodegenerative disease is still incomplete.

5.1.4.3 α -Syn enhances oxidative stress

One point of consensus is that oxidative stress clearly has a stimulatory effect on BACE1-mediated processing of APP. α -Syn is strongly linked to oxidative stress. Enhanced oxidative stress has been demonstrated in α -syn overexpressing cell models, measured by enhanced free radical production and levels of antioxidant protein glutathione (Hsu et al. 2000; Junn & Mouradian 2002). Furthermore, in α -syn transgenic mice, elevated transcription of Nrf2-regulated antioxidant enzymes has been detected, indicating a response to oxidative stress (Béraud et al. 2013). Cremades et al. (2012) attribute oxidative stress to α -syn oligomers, which they created recombinantly and applied to α -syn transgenic rat neurons. Cytoplasmic, but not mitochondrial, ROS production was induced by oligomers, but not monomers or fibrils (Cremades et al. 2012). Iron and copper chelators are able to block the α -syn oligomer-induced oxidative stress of transgenic rat neurons, and additionally iPSC-derived SNCA triplication human neurons (Deas et al. 2016). The clear implication is that the effect of α -syn aggregates is mediated by metals. Supporting this, α -syn is a ferrireductase and uses copper redox cycling to aid iron reduction. Fe(II) can produce hydrogen peroxide through the Fenton reaction if not adequately sequestered (Davies, Moualla, et al. 2011; Davies, Wang, et al. 2011). Hydrogen peroxide by copper-bound α -syn has been detected independently *in vitro* (C. Wang et al. 2011).

5.1.4.4 α -Syn indirectly promotes mitochondrial production of free radicals

The mitochondrial respiratory chain is responsible for most of the free radical production in cells, and this increases if mitochondrial homeostasis is disturbed. Impairs mitochondrial function in α -syn transgenic mice is apparent in the absence of α -syn toxic oligomers or insoluble aggregates (Sarafian et al. 2013). Several α -syn models have been identified to

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contain mitochondria with disturbed respiration (Devi et al. 2008; Luth et al. 2014), fragmented networks (Nakamura et al. 2011), and reduced import of respiratory substrates through the VDAC and TOM40 channels (Rostovtseva et al. 2015; Bender et al. 2013). Yet it appears increasingly likely that ER calcium stores mediate the effect of α -syn on mitochondrial function (Su et al. 2010). Mitochondria are coupled to ER membranes, where they take up ER-released calcium in order to sense and respond to local energy demands. High calcium release stimulates oxidative phosphorylation and free radical production in ER-tethered mitochondria (Hayashi et al. 2009). Wildtype α -syn is localised to sub-domains of the ER membrane that are tethered to mitochondria (Guardia-Laguarta et al. 2014). Indeed, increased calcium transfer from the ER to mitochondria has been shown to occur in α -syn overexpressing cells (Calì et al. 2012). α -Syn-induced free radical production in yeast can be prevented by depleting ER calcium stores, through genetic reduction of functioning PMR1. PMR1 is a Ca^{2+} -ATPase transporter of the Golgi and ER (Büttner et al. 2013).

5.1.5 Endoplasmic reticulum stress

5.1.5.1 BACE1 translation is enhanced by the eIF2 kinase PERK

Translation of BACE1 is controlled by the phosphorylation status of the translation initiation factor eIF2 α , as discussed previously. A number of eIF2 kinases phosphorylate eIF2 α in response to different intracellular stress pathways, including PKR, GCN2 (general control non-derepressible 2 kinase), HRI (heme-regulated inhibitor kinase), and PERK (PKR-like ER kinase) (O'Connor et al. 2008). PKR has been associated with oxidative stress-mediated translational regulation of BACE1 expression (Mouton-Liger et al. 2012). HRI could be involved in glutamatergic stimulation of BACE1 expression in hippocampal neurons, activated in response to nitrous oxide production (ILL-Raga et al. 2015). GCN2 is thought to be activated by nutrient deprivation, and is not thought to be important in animal models of AD. Genetic depletion of GCN2 in 5xFAD AD model mice actually resulted in enhanced eIF2 α phosphorylation and BACE1 protein expression. The unexpected result was discovered to coincide with over-activation of PERK, which only occurred in GCN2^{-/-} 5xFAD mice and not GCN2^{-/-} mice (Devi & Ohno 2013). PERK is localised to the ER membrane and is activated by ER stress, where ER chaperone-PERK complexes dissociate to deal with an excess of misfolded protein. PERK phosphorylation of eIF2 α is a key signalling pathway of the 'unfolded protein response' (Hoozemans et al. 2012). The importance of PERK to elevated BACE1 expression was subsequently confirmed, using the 5xFAD mouse model of AD. In PERK^{+/-} 5xFAD mice, the genetic depletion of PERK restores BACE1 expression almost to wildtype levels (Devi & Ohno 2014). On the other hand, in a different AD mouse model the

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conditional knockout of PERK did not reduce BACE1 expression. APP-PS1 mice with reduced PERK expression exhibited less eIF2 α phosphorylation in the hippocampus and prefrontal cortex, but no change to BACE1 (Ma et al. 2013). Ostensibly, this would support the study showing no effect of eIF2 α genetic depletion upon BACE1 expression (Sadleir et al. 2014). This conclusion is questionable, since the APP-PS1 mouse does not have higher BACE1 expression than the wildtype mouse to start with, in contrast with the aggressive 5xFAD model (Ma et al. 2013; Devi & Ohno 2014).

5.1.5.2 α -Syn causes ER stress, resulting in activation of PERK

ER stress is a ubiquitous feature of protein misfolding diseases. It is therefore unsurprising that PERK phosphorylation and other markers of the unfolded protein response are higher in PD and AD brains relative to age-matched controls (Hoozemans et al. 2012; Hoozemans et al. 2007; Selvaraj et al. 2012). In the synucleinopathies, α -syn misfolding may play a role in perpetuating ER stress. A study of PERK activation in PD brains showed that phosphorylated PERK was only present in neurons with α -syn immunostaining, although the majority of α -syn-positive neurons were free of detectable phosphorylated PERK (Hoozemans et al. 2007). ER stress has been studied more extensively in cell and animal models of synucleinopathy. Wildtype α -syn, in addition to more easily misfolded mutant forms such as A53T or 1-120, commonly induces ER stress in transgenic neuronal cell lines with stable or inducible expression (Belal et al. 2012; Smith et al. 2005; Bellucci et al. 2011; Colla et al. 2012). This appears to be accompanied by activation of PERK signalling, where studied (Bellucci et al. 2011). Wildtype and A53T α -syn transgenic rodents also demonstrate increased activation of the PERK-eIF2 α signalling pathway in two independent studies (Gorbatyuk et al. 2012; Belal et al. 2012). A third did not detect enhanced eIF2 α phosphorylation, instead finding that the XBP-1 branch of the unfolded protein response was activated (Colla et al. 2012). It would be unwise to interpret too much from individual studies, because the unfolded protein response is complex and dynamic. The relative activation of PERK-eIF2 α and the IRE-1-XBP-1 pathways can vary substantially between experimental systems and over time (Hetz et al. 2015).

5.1.5.3 α -Syn- induced ER stress may arise from defective ER-Golgi transport

Delayed or blocked ER-Golgi transition has been uncovered in studies of wildtype α -syn overexpressing neuronal cell and yeast models (Thayanidhi et al. 2010; Oaks et al. 2013; Cooper et al. 2006). One outcome may be reduced plasma membrane localisation of dopamine transporter DAT, instead accumulating within ER microsomes of α -syn neuroblastoma cells (Oaks et al. 2013). In yeast models, α -syn expression dose-dependently impairs ER-Golgi

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trafficking and activates ER stress and growth arrest. Interestingly, a reduction in α -syn toxicity was achieved by overexpressing genes that promote ER-Golgi transition, particularly orthologs of Rab-GTPases Rab1, Rab3a, and Rab8a (Cooper et al. 2006; Gitler et al. 2008). α -Syn may directly interact with and sequester Rab protein. Rab8a, a Rab-GTPase essential for trans-Golgi transport, co-immunoprecipitates with α -syn from A30P transgenic mouse brain homogenates (Dalfó et al. 2004). NMR studies show Rab8a binding directly to the C-terminal tail of α -syn, and overexpression of its ortholog in α -syn transgenic *Drosophila* was neuroprotective (Yin et al. 2014). Disruption of ER-Golgi transition could be through mislocalisation of Rab-GTPases, but there is also evidence that α -syn directly impacts vesicle docking or fusion through SNARE complexes. α -Syn binds to ER/Golgi SNAREs *in vitro* (Burré et al. 2014; Burré et al. 2010; Thayanidhi et al. 2010). Although non-aggregated α -syn is an aid to SNARE-complex formation, large toxic oligomers of α -syn appear to inhibit SNARE-mediated vesicle docking (Burré et al. 2010; Choi et al. 2013).

5.2 Aims

It is clear from the literature that there are a number of likely routes by which α -syn overexpression can be hypothesised to impact BACE1 levels in a cell. The aim of this chapter is to uncover the cellular pathway that drives elevated BACE1 expression in α -syn-overexpressing SH-SY5Ys. Intracellular pathways, selected from the literature, were probed for their contribution to BACE1 expression, and compared in α -syn SH-SY5Ys with empty vector cells. Protein clearance, calcium homeostasis, oxidative stress, and ER stress will separately be considered for their likelihood of being involved in the mechanism.

The following experiments will seek to answer a number of questions, which will be referred to in the discussion:

- Is there impaired degradation of BACE1 in α -syn cells, and is this likely to be responsible?
- Does reducing intracellular calcium release in α -syn cells restore BACE1 protein levels?
- Is there enhanced oxidative stress in α -syn cells?
- Could BACE1 expression be upregulated by transcription factors NF- κ B or AP-1, in α -syn cells?
- Is there increased activation of eIF2 α phosphorylation in α -syn cells, and could this be upregulating BACE1 translation?
- Does PKR mediate translational upregulation of BACE1 in response to oxidative stress in α -syn cells?

5.3 Results

5.3.1 Perturbation of protein degradation pathways leads to higher accumulation of BACE1 in α -syn SH-SY5Ys

Elevated BACE1 protein levels in WT α -syn SH-SY5Ys could be a result of increased expression. However, there is another possibility, which is that degradation of BACE1 and/or other proteins is impaired. BACE1 is known to be degraded by macroautophagy and by the ubiquitin-proteasome system. In some cell models α -syn has been shown to inhibit the proteasome, but no link to impaired macroautophagy has been made in the literature. The hypothesis that α -syn reduces BACE1 degradation was tested by incubating WT α -syn and empty vector SH-SY5Ys with chemical inhibitors of proteasome and lysosome function. Proteasomal degradation was inhibited with clastolactacystin- β -lactone (CL β L), and lysosomal acidification was inhibited with ammonium chloride (NH₄Cl). BACE1 protein levels were measured by western blotting. Accumulation of BACE1 in response to an inhibitor would indicate that that process is indispensable for BACE1 degradation. The magnitude of accumulation is controlled by the existing rate of BACE1 clearance, but also the rate of BACE1 synthesis. To control for changes in BACE1 synthesis, the protein synthesis inhibitor cycloheximide (CHX) was included. CHX alone should lead to decreased BACE1 levels, due to clearance, and when combined with CL β L and NH₄Cl there should be no change in BACE1 levels. However, CHX failed to prevent BACE1 accumulating in the presence of combined autophagy and proteasome inhibitors over the six-hour experiment, which means that translation was not inhibited. The control for protein synthesis was therefore a failure, which affects the conclusions of the experiment.

In empty vector cells, proteasome and lysosomal inhibitors were used individually and in combination to set a benchmark of how BACE1 levels respond. As expected, analysis of variance indicates that BACE1 protein levels in empty vector cells differed significantly between the treatment groups (F value= 6.79 for 5 and 12 degrees of freedom, $p < 0.01$). The significant changes, calculated by a post-hoc Tukey HSD test, are outlined in Figure 5.1a. An important finding is that lysosome inhibition and proteasome inhibition on their own did not significantly induce BACE1 accumulation. However, combined proteasome and autophagy inhibition caused a very large accumulation of BACE1. The simplest explanation for this is that BACE1 can be degraded by either the lysosome or the proteasome, within SH-SY5Ys, and one will compensate for the other if inhibited. Yet the lysosome is likely to be the preferred and more effective route, since its inhibition appeared to lead to a small accumulation of BACE1.

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The treatments were run in parallel with WT α -syn cells, to discover whether proteasome/lysosomal inhibitors had a similar effect on BACE1 protein levels. Between the treatment groups there were once again significant differences in BACE1 protein levels, shown by analysis of variance (F value= 7.31 for 5 and 12 degrees of freedom, $p < 0.01$). Figure 5.1b illustrates that the single most significant result was the ability of lysosome inhibitor ammonium chloride (NH_4Cl) to induce huge BACE1 accumulation, greater than any other treatment. On average, NH_4Cl had a greater effect upon BACE1 levels in WT cells (~2x) than empty vector cells (~1.5x). As well as being more sensitive to lysosome inhibition, WT cells appear to be slightly sensitive to proteasome inhibition, resulting in a small increase in BACE1. This was not significant in post-hoc tests, but appears distinct in Figure 5.1b. Note that in combination, lysosome and proteasome inhibition did not further potentiate BACE1 levels. Potentially the combined inhibition may have led to high cell death in the WT α -syn cells, but cell viability was not investigated.

In summary, WT α -syn cells appear more sensitive to both proteasome and lysosome inhibition. Most likely the latter has the greatest effect because BACE1 is preferentially degraded by the lysosome. There are two possible interpretations of the distinctive response: either (i) increased BACE1 protein synthesis leads to it accumulating faster when protein degradation is perturbed, or (ii) lysosomal and proteasomal degradation are both impaired. The protein synthesis control did not work, so the former cannot be ruled out. Without further investigations of autophagic or proteasomal health it is not possible to confirm the latter. Thus the experiment once again highlights the influence of α -syn upon BACE1 levels, but is inconclusive as to the mechanism.

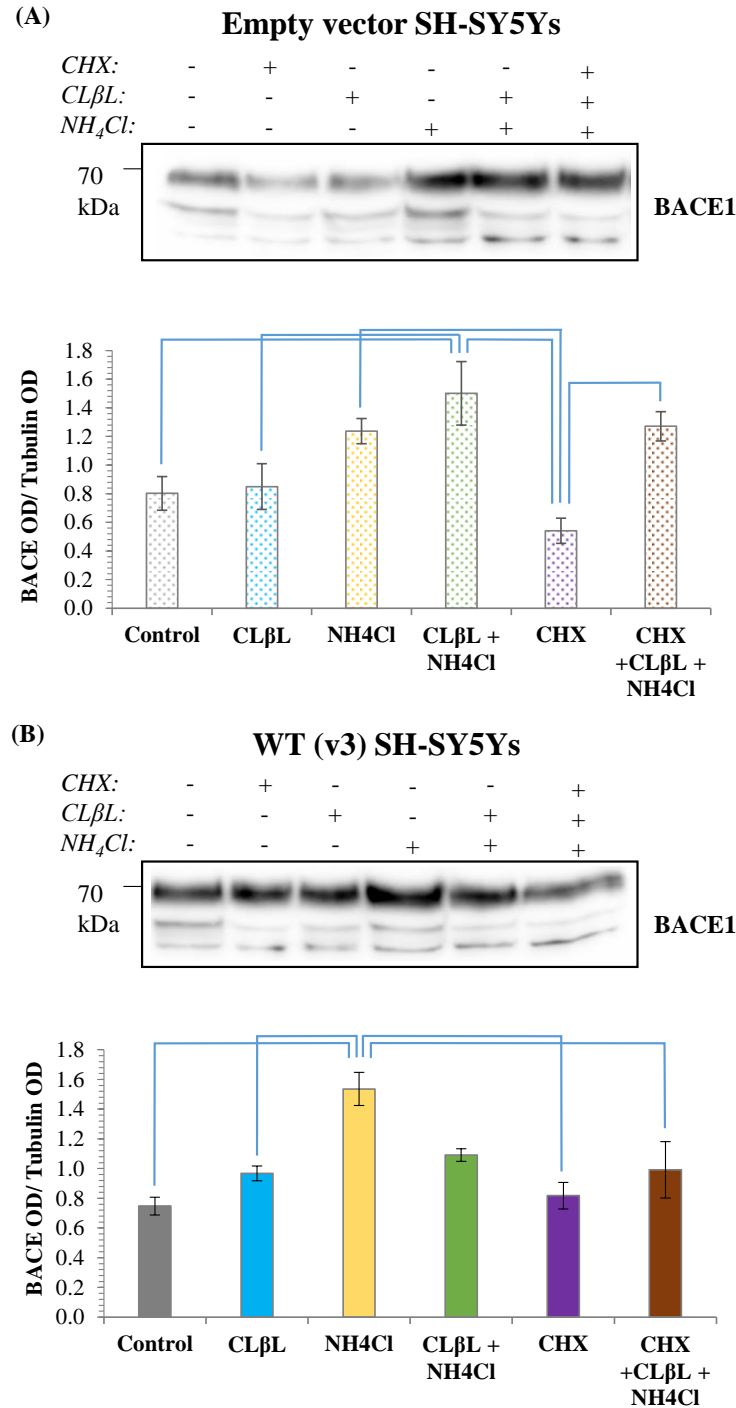


Figure 5.1 Accumulation of BACE1 protein with proteasome and lysosome inhibitors.

(A) Empty vector SH-SY5Ys, (B) WT α -syn SH-SY5Ys. Protein synthesis was inhibited with 100 μ g/ml cycloheximide (CHX), proteasomal degradation was inhibited with 20 μ M clastolactacystin- β -lactone (CL β L), and lysosomal acidification was inhibited with 2 mM NH₄Cl, all treatments for 6 hours. Whole cell lysates were tested for BACE1 and α -tubulin by western blotting. Mean BACE1 OD: α -tubulin OD for 3 independent experiments \pm S.E. Probability values calculated by one-way ANOVA with post-hoc Tukey's HSD, with all significant ($p < 0.05$) results indicated by connecting lines.

5.3.2 BACE1 protein expression is not potentiated by increased calcium signalling

α -Synuclein is known to strongly influence the balance of intracellular calcium levels in cells where it is over-expressed (Hettiarachchi et al. 2009). In particular, a recent study showed that α -syn expression dose-dependently increases the release of calcium ions from intracellular stores in yeast strains (Caraveo et al. 2014). Calcium is also known to play a role in the induction of BACE1 transcription, through downstream signalling pathways. Calcium spikes trigger calcineurin-mediated activation of transcription factors NFAT3/4 that bind the BACE1 promoter (Mei et al. 2015; Jin et al. 2012), and also a poorly-defined effect mediated by calpains (Liang et al. 2010). If α -syn increases the tendency of intracellular calcium stores to be released, then it could promote BACE1 expression. To support this hypothesis, the effect of calcium on BACE1 needed to first be confirmed. Chemical inducers of intracellular calcium, predicted to increase BACE1 expression, and chemical inhibitors of calcium release or signalling, predicted to reduce BACE1 expression, were used.

BACE1 protein levels were measured in WT α -syn SH-SY5Ys following 2 hours of chemical or drug treatment. This was sufficient time to see changes to BACE1 transcription or translation, but short enough that cell viability was not severely reduced by calcium toxicity. Intracellular calcium was increased by either the calcium ionophore A23187 (Jin et al. 2012), or by potassium chloride (KCl) treatment, which depolarises the plasma membrane and activates voltage-gated Ca^{2+} channels (Sousa et al. 2013). Small rises in intracellular calcium activates ryanodine receptors on the ER, which are calcium-activated Ca^{2+} channels, leading to release of intracellular calcium stores. This process was inhibited by treatment with dantrolene, which blocks activation of ryanodine receptors. Another drug, FK-506, was used to inhibit calcineurin activation. After 2 hours, no individual drug treatment significantly altered BACE1 levels compared with the control (Figure 5.2a). Yet collectively the drug treatments did significantly modulate BACE1, shown by analysis of variance (F value= 5.33 for 4 and 15 degrees of freedom, $p < 0.01$). Post-hoc analysis reveals a pattern whereby the calcium signal-inducing treatments, KCl and A23187, provided significantly different BACE1 expression to the calcium signal-suppressing treatments, dantrolene and FK-506. Unexpectedly, the pattern is in opposition to the hypothesis, because calcium signal suppressors resulted in greater BACE1 protein expression than calcium signal inducers.

The effects of calcium-modulating compounds on BACE1 were studied in this instance within WT α -syn SH-SY5Ys, because it was expected that the elevated BACE1 levels would more clearly show calcium-induced differences. Empty vector cells were not studied to the same extent, but one compound, dantrolene, was used for comparison with WT α -syn cells.

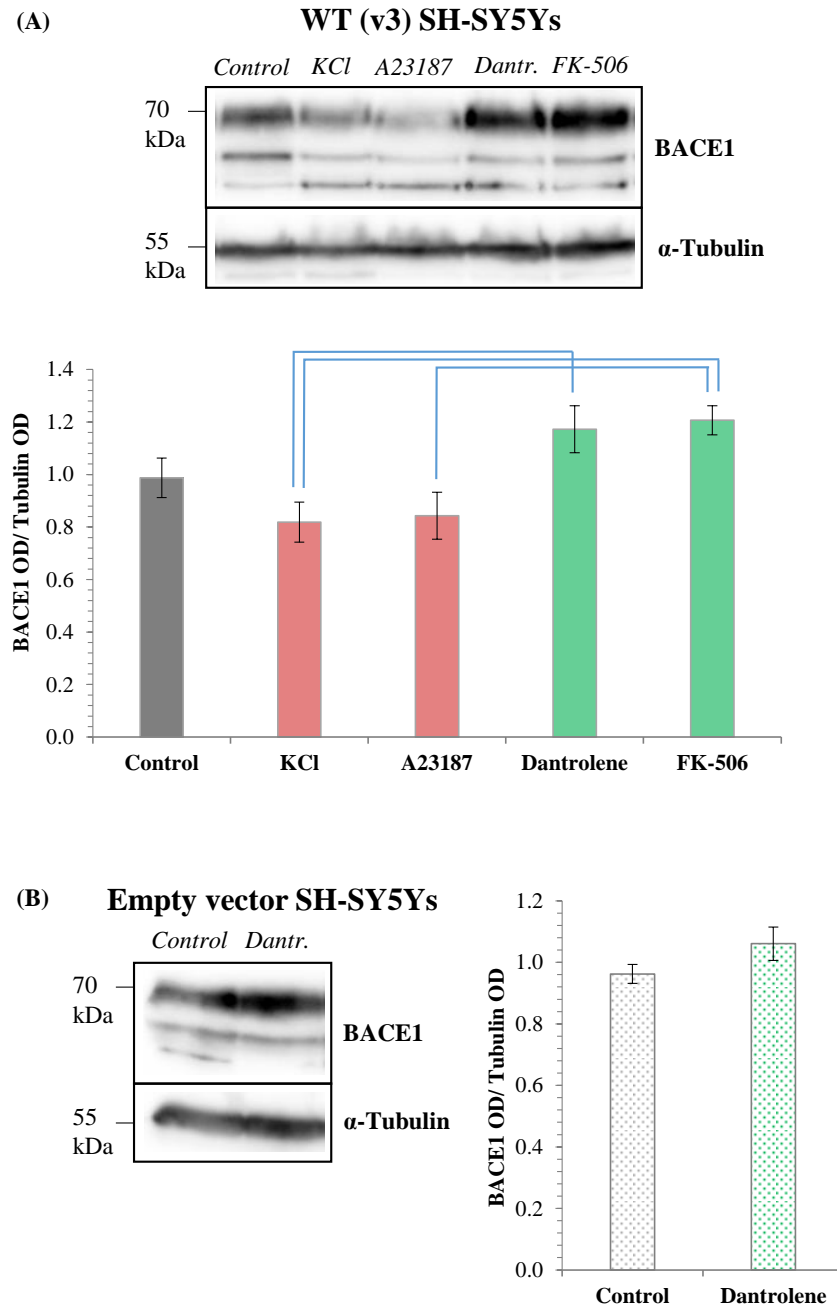


Figure 5.2 Intracellular calcium appears to negatively regulate BACE1 protein levels.

(A) WT α -syn SH-SY5Ys treated with pharmacological and chemical modulators of intracellular calcium, for 2 hours. Depolarising agent potassium chloride (KCl), 50 μ M, and ionophore A23187, 1 μ M, were used to depolarise the plasma membrane and increase calcium influx into the cell. Dantrolene, 10 μ M, was used to inhibit calcium-induced calcium release from the ER. FK-506, 1 μ M, was used to inhibit calcium-activated calcineurin signalling. **(B)** Empty vector SH-SY5Ys treated with 10 μ M dantrolene for 2 hours. Whole cell lysates were tested for BACE1 and α -tubulin by western blotting. Mean BACE1 OD: α -tubulin OD for 4 independent experiments \pm S.E. Probability values calculated by one-way ANOVA with post-hoc Tukey's HSD, with all significant ($p < 0.05$) results indicated by connecting lines.

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BACE1 levels were slightly enhanced in dantrolene-treated empty vector cells (Figure 5.2b). The difference is not significant, but follows the same direction as WT α -syn SH-SY5Ys. It seems likely, therefore, that α -syn does not increase BACE1 expression through changes in intracellular calcium.

5.3.3 *WT α -syn increases oxidative stress*

BACE1 protein expression is known to be upregulated by a variety of oxidative stressors, so it was hypothesised that over-expressed α -syn may be acting as an oxidative stress generator. α -Syn is a copper-binding protein with iron-reducing activity (Davies, Moualla, et al. 2011), and has been associated with oxidative stress in the literature. For example, α -syn potentiates copper-induced or dopamine-induced oxidative stress (Xu et al. 2002). Basal levels of oxidative stress in α -syn overexpressing cells are of more relevance to this thesis, but have not been described in the literature. To measure oxidative stress in α -syn SH-SY5Ys, the cells were loaded with a fluorescent molecular probe for hydrogen peroxide (CM-H₂DCFDA) and monitored for 60 minutes (Figure 5.3a). α -Syn significantly increased basal levels of hydrogen peroxide production, although the effect is small. In the literature, the redox activity of α -syn negatively correlates with its α -helical folding. Theoretically, disruption of α -syn folding with mutations could therefore enhance oxidative stress (Zhou et al. 2013). To test this hypothesis, Δ 2-9 and E46K mutant α -syn overexpressing SH-SY5Ys, were additionally assayed for oxidative stress (Figure 5.3a). Compared with WT α -syn SH-SY5Ys, neither significantly increased oxidative stress, yet hydrogen peroxide production appeared to be higher in Δ 2-9 α -syn cells.

A different ferrireductase enzyme, Steap3, was compared with α -syn, to see whether reduced iron production would have a measurable effect on oxidative stress in cells. Steap3 was previously generated by cloning the human Steap3 sequence (RefSeq accession number NG_042823.1) into a pcDNA3.1(+) vector, and stably transfected into SH-SY5Ys (Figure 5.3b). Steap3 overexpression led to more than a doubling of the rate of hydrogen peroxide production (Figure 5.3a). Ferrireductase activity can be inferred to significantly affect ROS production in cells, and therefore could potentially explain oxidative stress in α -syn SH-SY5Ys.

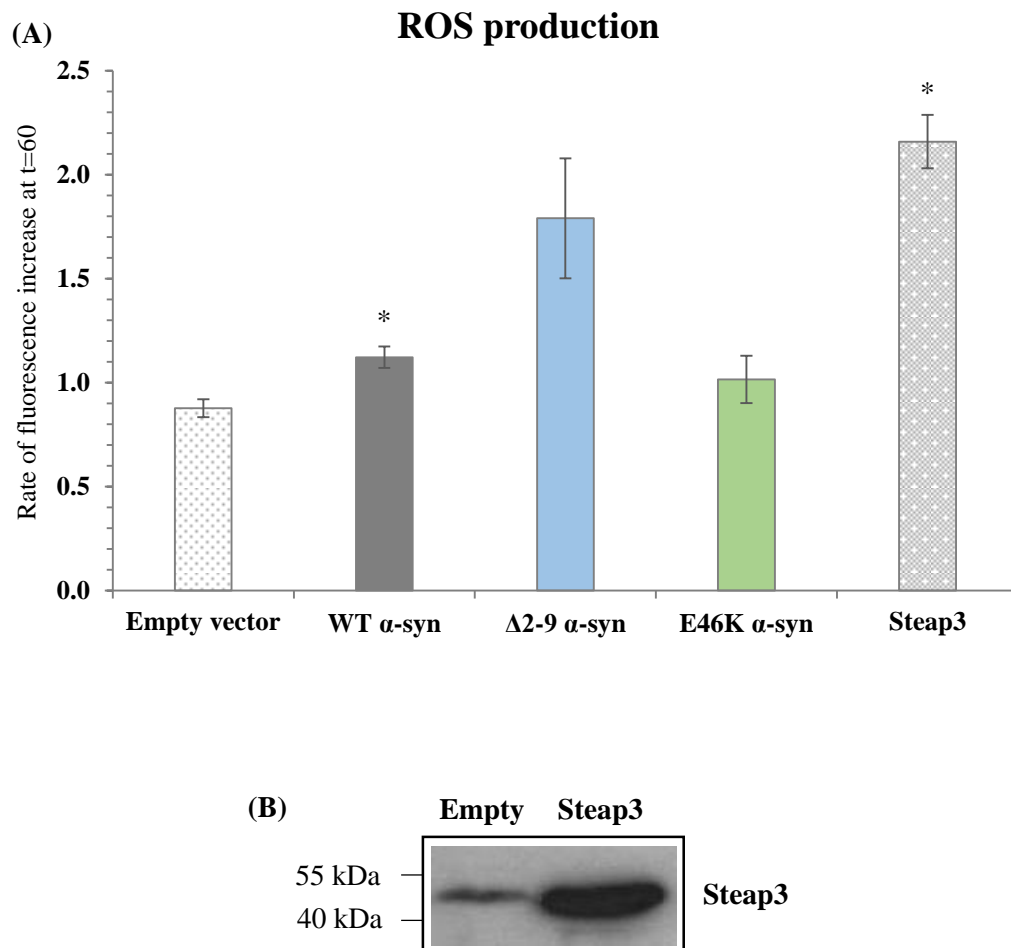


Figure 5.3 Overexpression of the ferrireductases α -syn and Steap3 increases oxidative stress. (A) Basal rates of ROS production in WT and mutant α -syn SH-SY5Ys, and Steap3 SH-SY5Ys. Oxidative stress was measured in live unstimulated cells using a fluorometric CM-H₂DCFDA probe for hydrogen peroxide. Mean rate of fluorescence increase \pm S. E. for 4 independent experiments. * $p < 0.05$ relative to the empty vector, no significant differences exist between mutant and WT α -syn lines, calculated by Student's t-test. (B) Steap3 expression in Steap3 SH-SY5Ys, compared with the empty vector. Western blotting was used to test whole cell lysates for Steap3 protein by Miss Ye Ding.

5.3.4 BACE1 expression is not upregulated by NF- κ B or AP-1 in SH-SY5Ys

A wealth of literature exists on the induction of BACE1 expression by oxidative stress. Two transcription factors appear to mediate oxidative stress-induced BACE1 transcription: NF- κ B and AP-1. To determine whether α -syn could potentially increase BACE1 levels through oxidative stress, the abilities of NF- κ B and AP-1 to induce BACE1 expression were investigated using small molecule inhibitors/activators.

In the literature, activation of NF- κ B leads to an induction of BACE1 transcription in SH-SY5Ys (Zheng et al. 2015), although has been shown to repress BACE1 transcription within neuronal cells in a previous study (Bourne et al. 2007). NF- κ B can be activated by protein kinase C (PKC) signalling, and this can be achieved experimentally using the small molecule phorbol myristyl acetate (PMA) (Holden et al. 2008). NF- κ B can be inhibited using sc-514, a selective small molecule inhibitor of I κ B kinase 2 (IKK2), the kinase that phosphorylates and activates NF- κ B. BACE1 expression was measured by western blotting. Incubation of WT α -syn SH-SY5Ys with PMA, to activate NF- κ B, did not lead to significant change in BACE1 protein levels (Figure 5.4a). Inhibition of NF- κ B with sc-514, however, significantly enhanced BACE1 protein expression in WT α -syn SH-SY5Ys (Figure 5.4b). This unexpected finding implies that NF- κ B negatively regulates BACE1 expression.

Activation of the transcription factor AP-1 by Jun kinase (JNK-1) has also been shown in the literature to upregulate BACE1 transcription (Tamagno et al. 2008). JNK-1 can be inhibited by the small molecule inhibitor SP600125. When applied to WT α -syn SH-SY5Ys, SP600125 appeared to increase BACE1 protein levels, although this was not statistically significant (Figure 5.4b). The similarity with the effect of NF- κ B inhibitor sc-514 is striking. One possible explanation for the apparent BACE1-enhancing effects of inhibiting these transcription factors, is that both NF- κ B and AP-1 may compete for the transcriptional co-activator CREB Binding Protein (CBP). Inhibiting NF- κ B, for instance, could allow more CBP to be available for binding AP-1 in active transcription complexes, and increase the activity of AP-1. Zheng et. al. characterised a pharmacological inhibitor of BACE1 transcription that partially acted by limiting the CBP available to NF- κ B, through activation of competing CREB/c-Jun pathways (Zheng et al. 2015). To investigate this, sc-514 and SP600125 were incubated together as well as separately. No enhancement or diminishment of BACE1 relative to the individual compounds was evident. The lack of additional BACE1 expression with combined sc-514 + SP600125 suggests that the individual effects of the drugs are not entirely independent. However, since the effects of the two inhibitors do not ‘cancel out’, there is no evidence that they are driven by strong competition for CBP. Therefore the most likely explanation is the simplest: NF- κ B and AP-1 both directly suppress BACE1 expression.

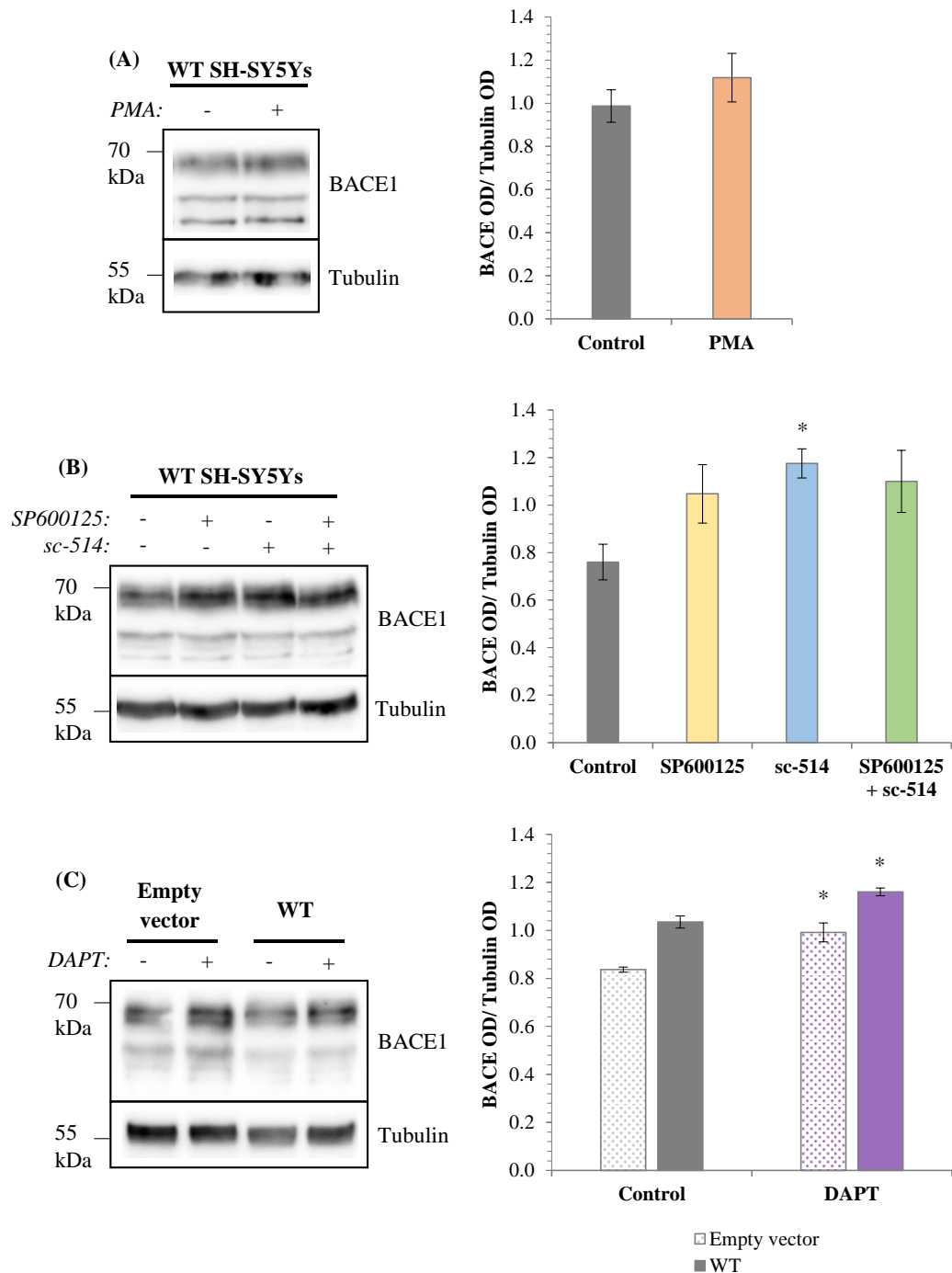


Figure 5.4 BACE1 protein expression is paradoxically increased by inhibitors of NF- κ B, JNK-1, and γ -secretase. (A) WT α -syn SH-SY5Ys incubated 2 hours with an activator of NF- κ B, PMA (phorbol 12-myristate 13-acetate; 1 μ M). (B) WT α -syn SH-SY5Ys incubated 2 hours with inhibitors of NF- κ B, sc-514 (50 μ M), and JNK-1, SP600125 (2 μ M). (C) Empty vector and WT α -syn SH-SY5Ys incubated 6 hours with a selective γ -secretase inhibitor, DAPT (2 μ M). Whole cell lysates were tested for BACE1 and α -tubulin by western blotting. Mean BACE1 OD: α -tubulin OD for 4 independent experiments \pm S.E. * $p < 0.05$ relative to control, calculated by one-way ANOVA with post-hoc Tukey's HSD.

γ -Secretase activity, potentiated by oxidative stress, has been proposed to drive BACE1 transcription through an AP-1-dependent route. Tamagno et al. showed that increased γ -secretase activity causes greater β -amyloid production, which activates JNK-1 signalling and AP-1 (Tamagno et al. 2008). Therefore, pharmacological inhibition of γ -secretase would be anticipated to have a similar effect as inhibiting JNK-1. DAPT was used to inhibit γ -secretase in both empty vector and WT α -syn SH-SY5Ys, and changes to BACE1 expression measured by western blotting (Figure 5.4c). Although basal levels of BACE1 are higher in the α -syn SH-SY5Ys, both lines respond to DAPT with significant BACE1 upregulation. This agrees with the findings that inhibiting oxidative stress-responsive transcription factors NF- κ B and AP-1 potentiates BACE1 expression. It is also clear that α -syn overexpression itself does not have a significant impact on the changes seen. To summarise, the apparently induced oxidative stress in WT α -syn SH-SY5Ys is unlikely to enhance BACE1 expression via the transcription factors NF- κ B and AP-1.

5.3.5 α -Syn overexpression enhances levels of eIF2 α phosphorylated at Ser-51

BACE1 is translationally repressed under normal conditions, since the 5'UTR of BACE1 mRNA is GC-rich and is predicted to form stable secondary structure (Lammich et al. 2004). An effective route to upregulating BACE1 expression would be to promote phosphorylation of eIF2 α , which enhances BACE1 translation (O'Connor et al. 2008). So far it has become apparent that WT α -syn increases BACE1 protein levels but decreases BACE1 promoter activation (Chapter 4), so an increase in translation was hypothesised. Studying BACE1 protein synthesis by itself is not straight-forward. However, phosphorylation of eIF2 α is easily detectable by western blotting, and could signal changes to BACE1 translation. Western blots of cell extracts from multiple WT α -syn SH-SY5Y lines were probed with a Ser51 phosphorylation-specific antibody for eIF2 α , and re-probed for total eIF2 α (Figure 5.5a). On average, the WT lines collectively had a significantly higher ratio of phosphorylated: total eIF2 α . The enhanced eIF2 α phosphorylation indicates potential de-repression of BACE1 translation.

Phosphorylation of eIF2 α was also measured in two truncated (Δ 2-9 & Δ NAC) and two disease-mutant (E46K & A53T) α -syn SH-SY5Y lines (Figure 5.5b). Δ 2-9 and A53T lines had significantly enhanced eIF2 α -phosphorylation when compared with WT (v1), but Δ NAC and E46K did not. This does not closely follow previously described patterns of BACE1 expression across the lines, since BACE1 protein levels were higher in Δ NAC and E46K than WT α -syn SH-SY5Ys.

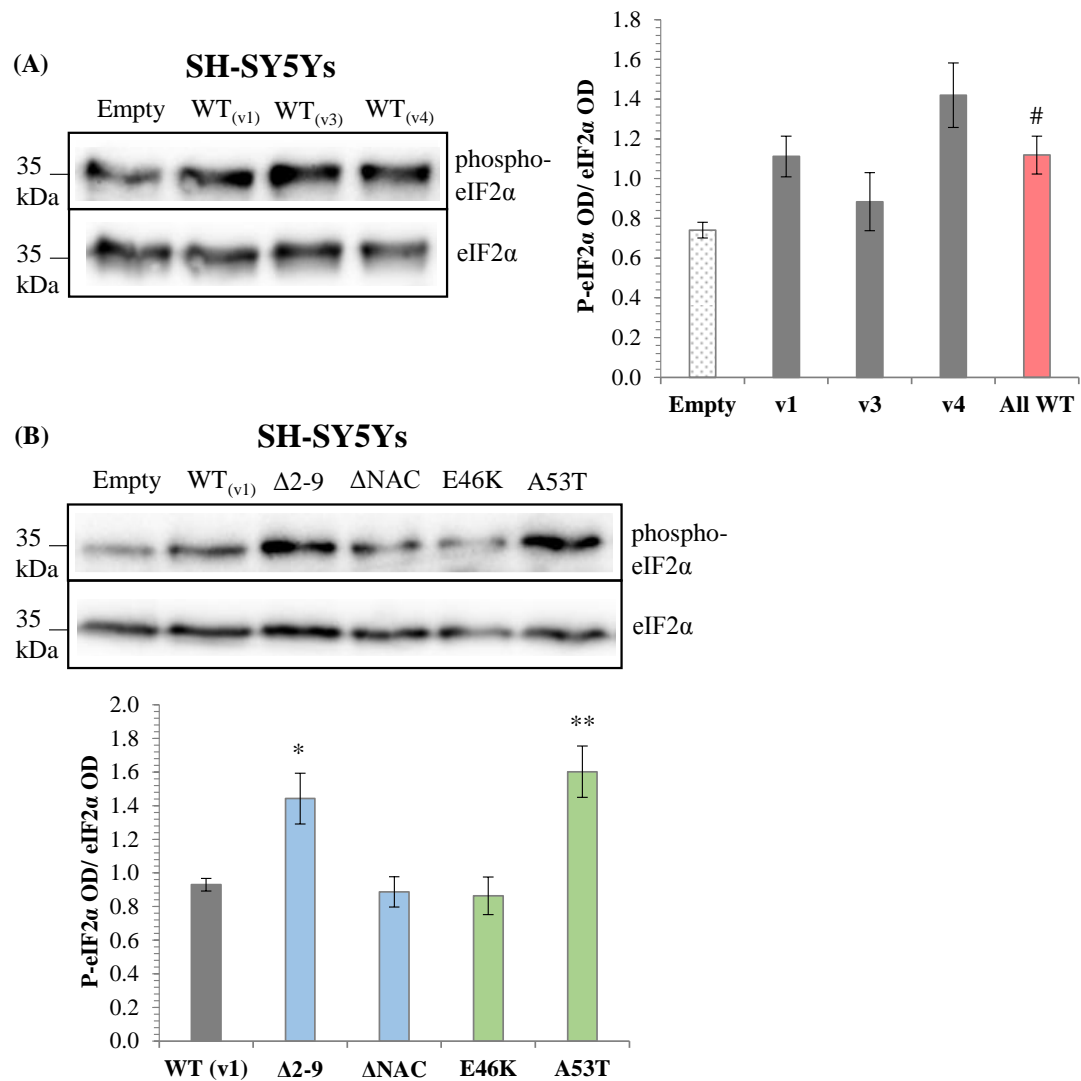


Figure 5.5 eIF2 α phosphorylation in SH-SY5Y lines. Levels of ser-51 phosphorylated eIF2 α in (A) WT α -syn SH-SY5Ys, (B) α -syn mutant SH-SY5Ys. Whole cell lysates were tested for phospho-eIF2 α and eIF2 α by western blotting. Mean phospho-eIF2 α OD: eIF2 α OD for 3-5 independent experiments \pm S.E. # $p < 0.05$ relative to empty vector, * $p < 0.05$, ** $p < 0.01$ relative to WT, calculated by pairwise t-tests with a Holm adjustment.

5.3.6 α -Syn does not affect basal or tunicamycin-induced eIF2 α phosphorylation

Phosphorylation of eIF2 α is induced by a number of different eIF2 kinases in response to cell stresses, and constitutive dephosphorylation is performed by the GADD34/PP1 complex. Prolonged block in translation due to phosphorylated eIF2 α results in cell death, thus a healthy cell line is unlikely to have constantly high phosphorylated eIF2 α (Hetz et al. 2015). However, elevated basal levels of eIF2 α phosphorylation have been previously reported in an overexpressing cell model of Alzheimer's disease, as a long-term adaptation to ER stress (Lewerenz & Maher 2009). Cell overexpression of α -syn in the literature induces ER stress (Belal et al. 2012; Bellucci et al. 2011; Colla et al. 2012; Smith et al. 2005), therefore it was hypothesised that overexpression of α -syn in SH-SY5Ys may cause a small chronic increase in eIF2 α phosphorylation. eIF2 kinase activity can be isolated with salubrinal, a selective inhibitor of eIF2 α dephosphorylation by GADD34/PP1 (Boyce et al. 2005). The WT (v3) and empty vector SH-SY5Ys were incubated with salubrinal for 0.5 and 1 hour, before western blotting for phosphorylated and total eIF2 α (Figure 5.6a). Levels of BACE1 protein were also measured in the same cell extracts (Figure 5.6b). As expected, levels of phosphorylated eIF2 α appeared to accumulate in empty vector cells (not statistically significant). BACE1 protein increased correspondingly. In WT α -syn SH-SY5Ys, phosphorylated eIF2 α did not significantly accumulate during salubrinal treatment, although BACE1 protein levels rose. It is likely that accumulation of phosphorylated eIF2 α did occur in salubrinal-treated WT cells, since BACE1 protein levels increased, and was perhaps subject to high noise. Yet for WT α -syn SH-SY5Ys to have elevated basal eIF2 kinase activity, one would expect a more robust response to salubrinal, in comparison to empty vector cells. This does not appear to be the case, and it is possible that basal eIF2 α phosphorylation is tightly regulated.

Another possible source of differences in eIF2 α phosphorylation between SH-SY5Y lines is a heightened sensitivity of α -syn cells to stress stimuli. Heightened sensitivity would mean that eIF2 kinases may be activated greater and for longer in response to any stressor. This theory was tested by incubating α -syn cells and empty vector cells with tunicamycin, which is a well-established ER stress inducer that activates the eIF2 kinase PERK (Harding et al. 2000). Levels of eIF2 α dephosphorylation were controlled for by simultaneously treating cells with salubrinal. WT α -syn SH-SY5Ys had higher levels of phosphorylated eIF2 α to empty vector cells when unstimulated, as noted previously. Tunicamycin appeared to induce eIF2 α phosphorylation in both lines, and co-incubation with salubrinal potentiated the effect, as expected (Figure 5.7). α -Syn appears to have little effect on the extent of eIF2 α phosphorylation under conditions of PERK stimulation. Therefore, there is no evidence that α -syn overexpression confers an enhanced sensitivity to ER stress.

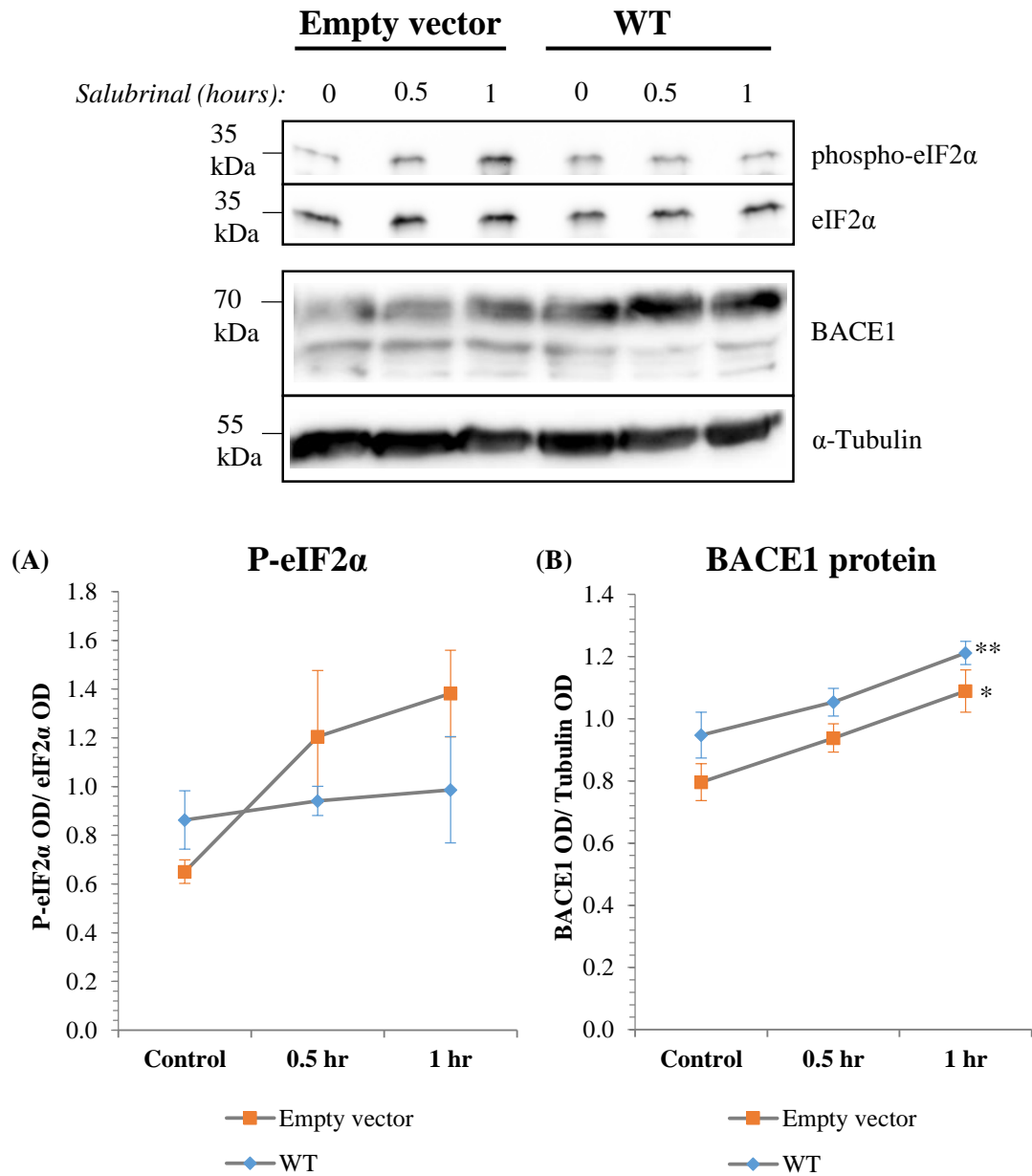


Figure 5.6 Salubrinal causes accumulation of phosphorylated eIF2 α and a consistent increase in BACE1 protein expression over time. Empty vector and WT α -syn SH-SY5Ys were treated with 20 μ M salubrinal for 0.5 or 1 hour, to inhibit de-phosphorylation of eIF2 α . (A) Levels of eIF2 α phosphorylation. Mean phospho-eIF2 α OD: eIF2 α OD for 6 independent experiments \pm S.E. (B) Concurrent expression of BACE1 protein. Mean BACE1 OD: α -tubulin OD for 6 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to control, calculated by one-way ANOVAs with post-hoc Tukey's HSD for the individual cell lines.

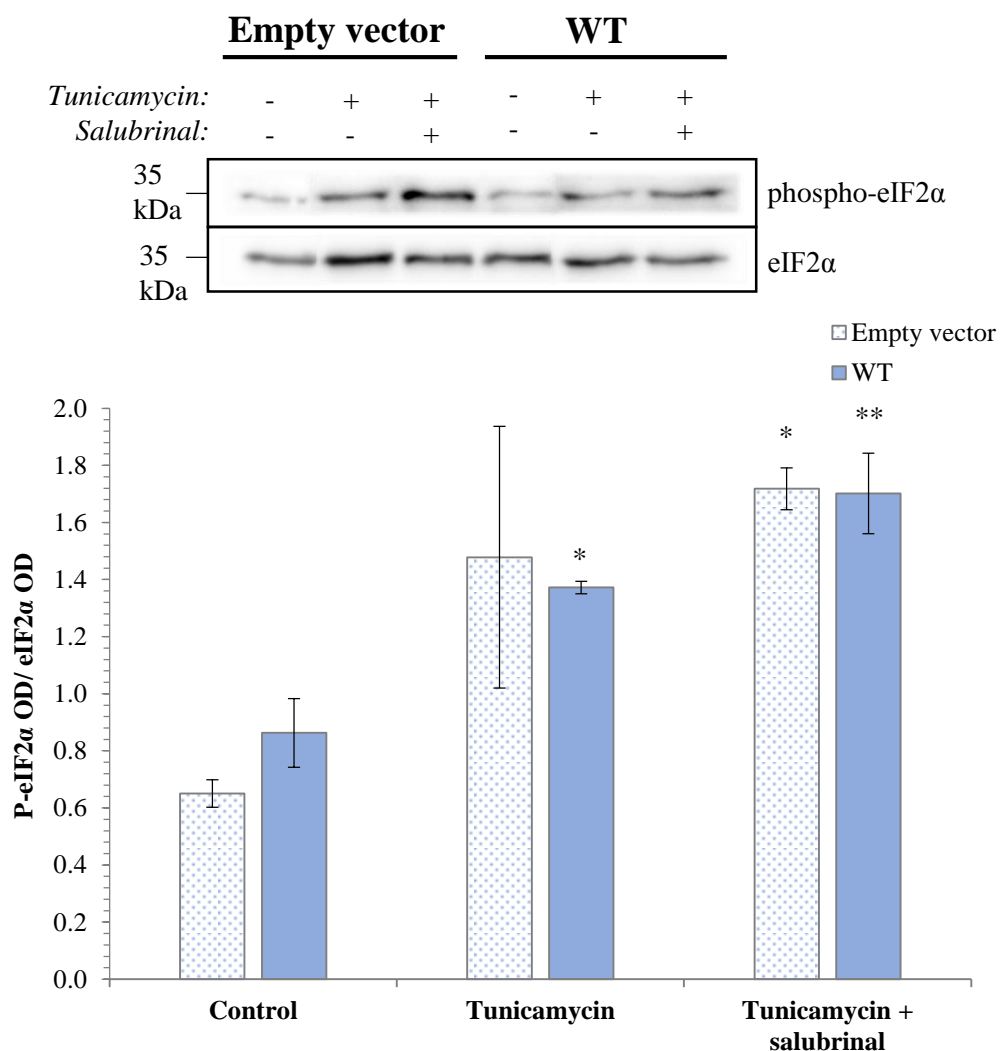


Figure 5.7 ER stressor tunicamycin induces eIF2 α phosphorylation, potentiated by salubrinal in SH-SY5Ys. Tunicamycin was used at 1 μ g/ml and salubrinal at 20 μ M, for 1 hour before lysis. Mean phospho-eIF2 α OD: eIF2 α OD for 3 independent experiments \pm S.E. * $p < 0.05$, ** $p < 0.01$ relative to control, calculated by one-way ANOVAs with post-hoc Tukey's HSD for the individual cell lines.

5.3.7 Pharmacological inhibition of the oxidative stress-activated eIF2 kinase 'PKR' does not reduce eIF2 α phosphorylation

So far there it has been shown that WT α -syn SH-SY5Ys have a greater proportion of eIF2 α phosphorylated at Ser-51, and that BACE1 expression will increase in response to a selective block of eIF2 α dephosphorylation. However, WT α -syn SH-SY5Ys do not appear to be more sensitive to activation of the eIF2 kinase PERK by ER stress stimuli. Other eIF2 kinases could be responsible for eIF2 α phosphorylation in α -syn cells. It was shown earlier in this chapter that α -syn overexpression causes higher production of ROS. Oxidative stress is known to activate the eIF2 kinase 'protein kinase R' (PKR). Therefore, it was proposed that PKR may be more activated in WT α -syn SH-SY5Ys, and drive BACE1 translation. A chemical inhibitor of PKR was obtained, as a cost-effective and quick method to test this hypothesis. If PKR had an important role, then its inhibition ought to reduce eIF2 α phosphorylation and BACE1 protein levels in WT α -syn SH-SY5Ys, but not empty vector cells. Empty vector and WT (v3) SH-SY5Ys were treated with high and low doses of PKR inhibitor for 2 hours (Figure 5.8). PKR inhibitor slightly reduced BACE1 expression, but the effect was not statistically significant, and not limited to the WT (v3) line. Furthermore, eIF2 α phosphorylation was strongly induced by the PKR inhibitor, rather than suppressed. Induced eIF2 α phosphorylation suggests that there is compensatory activation of other eIF2 kinases. The fact that this does not *increase* BACE1 expression suggests that PKR may be important for BACE1 expression in SH-SY5Ys. Further concentrations of PKR inhibitor would be necessary to ascertain whether WT (v3) SH-SY5Ys are more sensitive than empty vector cells.

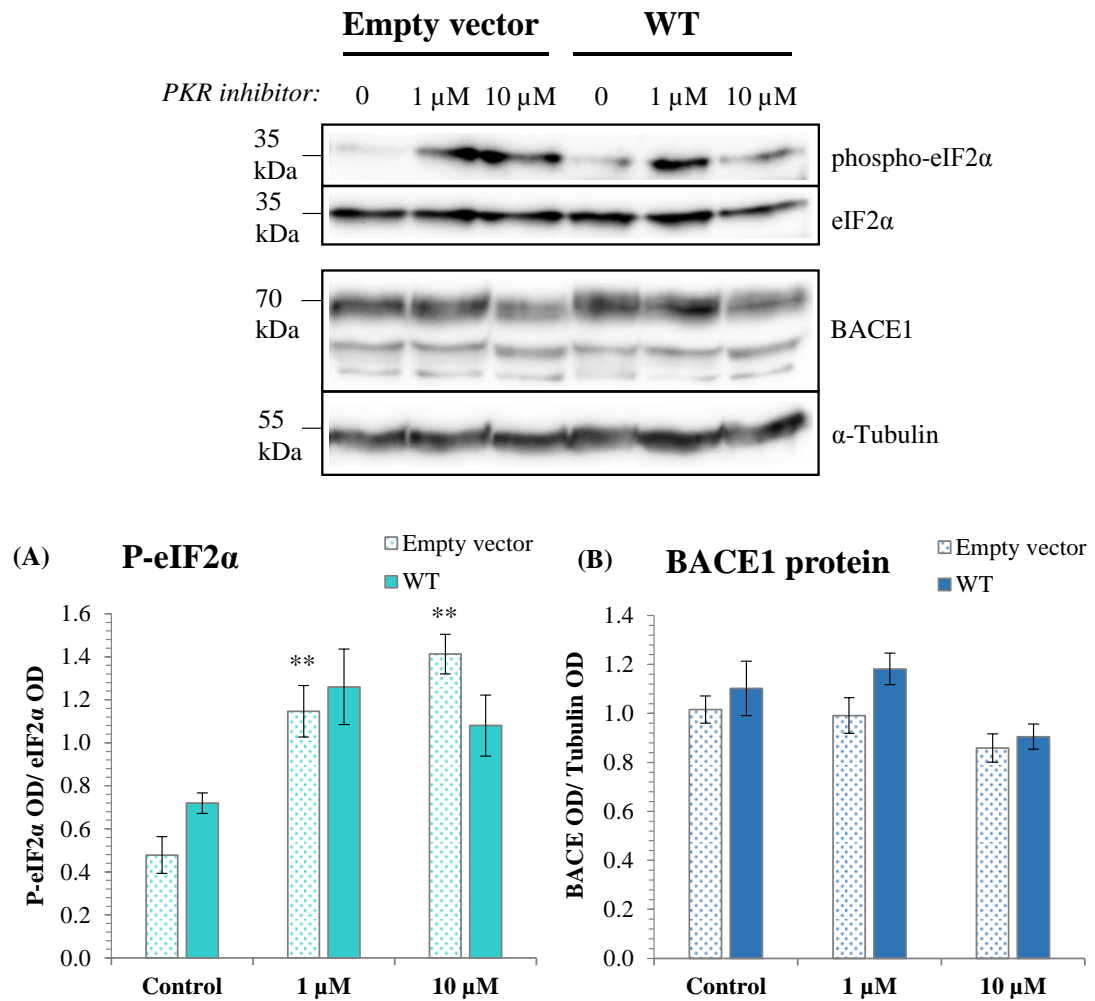


Figure 5.8 PKR inhibitor induces eIF2 α phosphorylation, and not BACE1 expression. Empty vector and WT α -syn SH-SY5Ys were treated with 1 μ M or 10 μ M PKR inhibitor for 2 hours. **(A)** Levels of eIF2 α phosphorylation. Mean phospho-eIF2 α OD: eIF2 α OD for 3 independent experiments \pm S.E. **(B)** Concurrent expression of BACE1 protein. Mean BACE1 OD: α -tubulin OD for 3 independent experiments \pm S.E. ** $p < 0.01$ relative to control, calculated by one-way ANOVAs with post-hoc Tukey's HSD for the individual cell lines. No significant differences in the WT SH-SY5Y data.

5.4 Discussion

α -Syn overexpression in a neuronal cell model correlates both with increased amyloidogenic processing of APP, and increased BACE1 expression, suggesting the existence of a novel pathway. Understanding the mechanistic details of this pathway could improve understanding of synucleinopathy diseases. The data presented in this chapter is a preliminary exploration of different lines of enquiry, using BACE1 protein expression as an easy readout. Indirect cellular mechanisms were chosen for investigation based on a strong body of literature supporting both their instigation by α -syn overexpression, and their effect upon upregulating BACE1 expression. The experiments and their results are summarised in Table 4.

The proposed indirect mechanisms are unlikely to exclusively alter BACE1 expression, since the activities of the β -, γ -, and α -secretases are linked by a complex web of signalling pathways. For example, calcium activates α -secretase. Activation of NMDA receptors, leading to high Ca^{2+} influx, is known to promote retention of ADAM10 at the plasma membrane, thus increasing α -secretase cleavage of APP (Marcello et al. 2013). Concurrently, transport of mature ADAM10 to the plasma membrane is rapidly induced, in a mechanism mediated by classical PKC signalling (Saraceno et al. 2014). Calcium may also affect the conformation and synaptic localisation of γ -secretase, enhancing A β 42 secretion at the synapse (Kuzuya et al. 2016). Oxidative stress also increases γ -secretase activity, by increased presenilin-1 transcription (Oda et al. 2010). In fact γ -secretase appears to be integral to initiating transcriptional upregulation of BACE1 in response to oxidative stressors (Tamagno et al. 2008). A γ -secretase inhibitor was used to specifically probe the contribution of γ -secretase to BACE1 expression in SH-SY5Ys. Surprisingly, this increased BACE1 protein levels rather than suppressing BACE1 expression, highlighting the complexity of secretase interactions. ER stress can halt γ -secretase mediated cleavage of APP, by stalling exit of proteins from the ER (Wiley et al. 2010). Paradoxically, ER stress also activates PERK, which is believed to increase BACE1 expression (Devi & Ohno 2014). As previously discussed, phosphorylated eIF2 α promotes BACE1 translation as part of a sub-set of stress-response proteins. γ -Secretase may be indirectly upregulated by phosphorylated eIF2 α , as well. In response to amino acid deprivation, γ -secretase transcription is transactivated by ATF4, an eIF2 α -inducible stress-response protein (Mitsuda et al. 2007).

Furthermore, the mechanisms chosen for investigation are by no means the only possibilities by which α -syn could affect APP. Two major areas have been omitted: (a) Direct protein interactions with α -syn; (b) Epigenetic influences of α -syn. Direct interactions between α -syn and BACE1 or APP were not investigated, since the main focus was a neuronal cell model of synucleinopathy. Interestingly, unbiased proteomics of α -syn interactors have

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Proposed mechanism	Compound treatment or manipulation	Hypothesised effect on BACE1 protein levels	Measured change to BACE1 protein levels	α -Syn specific effect?
Proteasome impairment	Clastolactacystin- β -lactone (proteasome inhibitor)	↑	ns	No
Autophagy impairment	Ammonium chloride (lysosome inhibitor)	↑	↑	Yes
Signalling from elevated intracellular $[Ca^{2+}]$	Potassium chloride (depolarisation agent)	↑	↓	?
	A23187 (Ca^{2+} ionophore)	↑	↓	?
	Dantrolene (inhibit RyR-mediated release of Ca^{2+} from ER)	↓	↑	No
	FK-506 (selective calcineurin inhibitor)	↓	↑	?
Signalling from elevated oxidative stress	Unstimulated measurement with CM- H_2DCFDA	-	-	Yes
	PMA (activator of classical PKC signalling and NF- κ B)	↑	ns	?
	sc-514 (selective inhibitor of I κ B kinase-2)	↓	↑	?
	SP600125 (selective JNK inhibitor)	↓	ns	?
	DAPT (γ -secretase inhibitor)	↓	↑	No
	PKR inhibitor	↓	ns	No
Stress-induced phosphorylation of eIF2α	Unstimulated measurement with Western blotting	-	-	Yes
	Salubrinal (selective inhibitor of eIF2 α dephosphorylation by Gadd34/PP1 complex)	↑	↑	No
	Compensatory increase in eIF2 α phosphorylation from PKR inhibitor	↑	ns	No

Table 4 Summary of Chapter 5 results. For individual experiments, the change to BACE1 predicted from the literature is shown, followed by the real result. The final column comments on whether the BACE1 change is potentiated by α -syn overexpression, i.e. α -syn specific, or the same in both α -syn and empty vector SH-SY5Ys. ns: not statistically significant. ?: not compared with empty vector cells, therefore the contribution of α -syn is unknown.

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identified APP as a hit, but not BACE1 (Mcfarland, Ellis, Markey, & Nussbaum, 2008). Direct protein-protein interactions could affect the co-localisation of APP and BACE1, alter the kinetics of secretase cleavage, or alter the stability and half-life of APP/BACE1. α -Syn could also affect gene transcription, in a way that indirectly affects APP processing. There is limited evidence to suggest that α -syn can bind to histone H3 and reduce H3 acetylation (Kontopoulos, Parvin, & Feany, 2006; Snead & Eliezer, 2014). Reduced activity of the p300 histone acetyltransferase has also been shown in α -syn overexpressing N2A cells, with the outcome of decreased PKC δ expression (Jin et al. 2011). PKC δ is a PKC isoform that potently activates α -secretase processing of APP (Yi et al. 2012). The rest of this discussion will focus upon answering the questions raised in the introduction, before making new hypotheses on the mechanism by which α -syn influences BACE1 expression.

5.4.1 Is there impaired degradation of BACE1 in α -syn cells, and is this likely to be responsible?

BACE1 is cleared by the proteasome and lysosome (Figure 5.9). A clear difference in the effectiveness of proteasomal and lysosomal degradation of BACE1 can be seen between α -syn and empty vector SH-SY5Ys. BACE1 protein levels accumulated significantly when α -syn cells were treated with an autophagy inhibitor, and slightly accumulated in the presence of a proteasome inhibitor. In contrast, empty vector cells only achieved significant BACE1 accumulation when both autophagy and the proteasome were inhibited together, with only a small response to the autophagy inhibitor alone. The apparently increased sensitivity of α -syn cells to inhibition of the proteasome and lysosome could be due to proteasome block or defective autophagy, or both. It is not possible to differentiate the two using this type of experiment, due to the cross-talk between the ubiquitin-proteasome system and autophagy. Proteasome inhibition causes autophagy induction, whereas autophagy inhibition impairs proteasome degradation as well (Korolchuk et al. 2009). BACE1 clearance as a whole relies more on autophagy, since it is a relatively long-lived membrane protein (Huse et al. 2000), and this is evident in higher response to autophagy inhibition than proteasome inhibition for both cell lines. Autophagy inhibition is thus more likely to make an impact on BACE1 levels. Furthermore, in the literature proteasome inhibition appears to be associated with α -syn aggregates (Tanaka et al. 2001; Stefanis et al. 2001), which have not been detected in the WT α -syn SH-SY5Ys used for this study. It is thus unlikely to be the proteasome that is impaired in α -syn SH-SY5Ys. An alternative explanation for the data is that there is a higher translation rate of BACE1 in α -syn SH-SY5Ys, which may cause it to accumulate faster where protein clearance is inhibited. This cannot be ruled out, since the protein synthesis inhibitor used as a

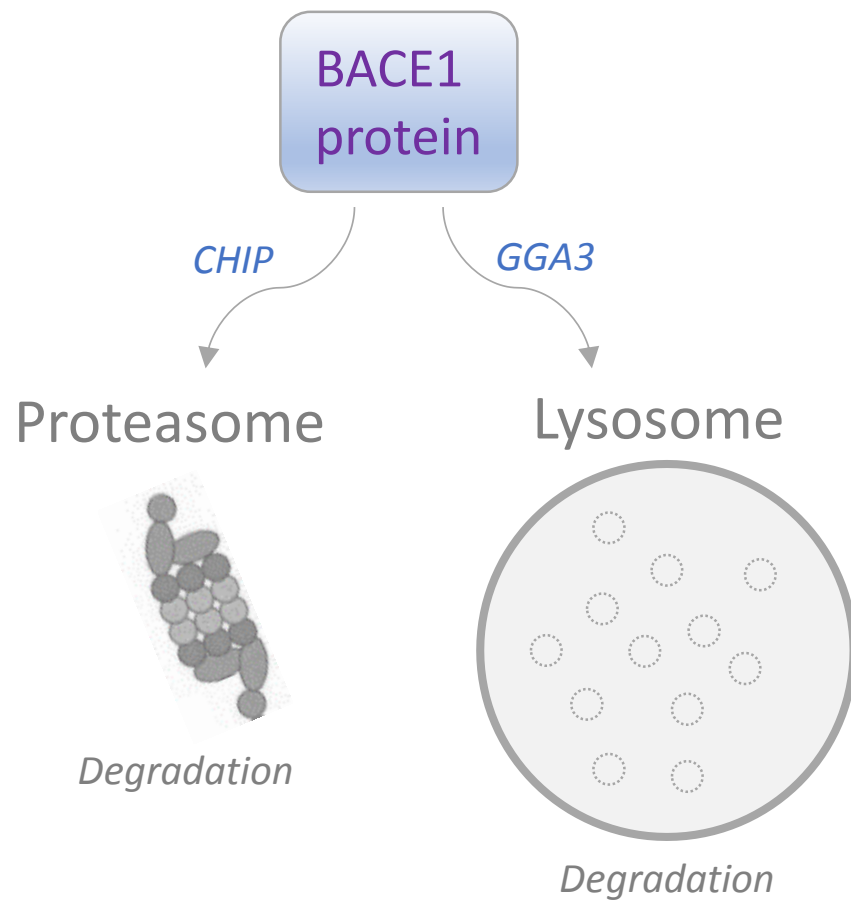


Figure 5.9 BACE1 protein levels can be enhanced by a reduction in degradation of BACE1 by the proteasome or lysosome. The E3 ligase CHIP binds and ubiquitinates BACE1, causing it to be targeted to the proteasome. GGA3 is an adaptor protein that delivers BACE1 to lysosomes from endosomal compartments.

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control was ineffective. A more detailed study of BACE1 intracellular localisation would be necessary to confirm whether less is trafficked to the lysosome. While the preliminary data is intriguing, there is insufficient evidence to conclude that impaired BACE1 degradation elevates BACE1 expression in α -syn cells.

5.4.2 Does reducing intracellular calcium release in α -syn cells restore BACE1 protein levels?

Increased intracellular calcium levels have been linked to α -syn overexpression in the literature (Caraveo et al. 2014; Furukawa et al. 2006). BACE1 transcription appears to be enhanced by calcium signalling, as illustrated in Figure 5.10 (Cho et al. 2008; Mei et al. 2015; Jin et al. 2012; Liang et al. 2010). It was therefore hypothesised that BACE1 expression in α -syn SH-SY5Ys would be reduced by inhibitors of ER calcium store release (dantrolene) and calcineurin signalling (FK-506). By the same logic, potassium chloride and A23187, which increase intracellular calcium levels, would be expected to increase BACE1 expression. In fact the complete opposite was found. Inhibiting calcium signalling increased BACE1 protein levels in α -syn SH-SY5Ys, and activating calcium signalling had a slight suppressive effect. The effect is not likely to be specific to α -syn cells, since dantrolene also appeared to enhance BACE1 expression in empty vector cells. Although calcium clearly has an impact on BACE1 expression, the effect appears to be one of negative regulation in SH-SY5Ys. This does not support a role for intracellular calcium as a positive mediator of α -syn-induced BACE1 expression.

5.4.3 Is there enhanced oxidative stress in α -syn cells?

Rates of hydrogen peroxide production in wildtype and mutant α -syn SH-SY5Ys were measured, to study oxidative stress. Basal levels of oxidative stress have not been previously described in the literature for any α -syn cell model, although copper and dopamine are known to induce an oxidative stress response (Xu et al. 2002). *In vitro* experiments appear to show hydrogen peroxide being produced from α -syn, dependent on its binding of copper, and the toxicity of α -syn application to cells can be prevented by catalase (C. Wang et al. 2011). α -Syn is suggested to be a ferrireductase, and uses bound copper to aid the redox-cycling of iron (Brown 2013; Peng et al. 2010). Considerably elevated levels of free cellular Fe(II) compared with Fe(III) have been demonstrated in WT α -syn SH-SY5Ys, which is likely to generate hydrogen peroxide through Fenton reactions (Davies, Moualla, et al. 2011). Oxidative stress was indeed significantly elevated in wildtype α -syn SH-SY5Ys, although the magnitude of the change was small. N-terminal truncation of α -syn appears to further elevate oxidative stress in cells. Although the effect of Δ 2-9 is not statistically significant, it is compelling due to the

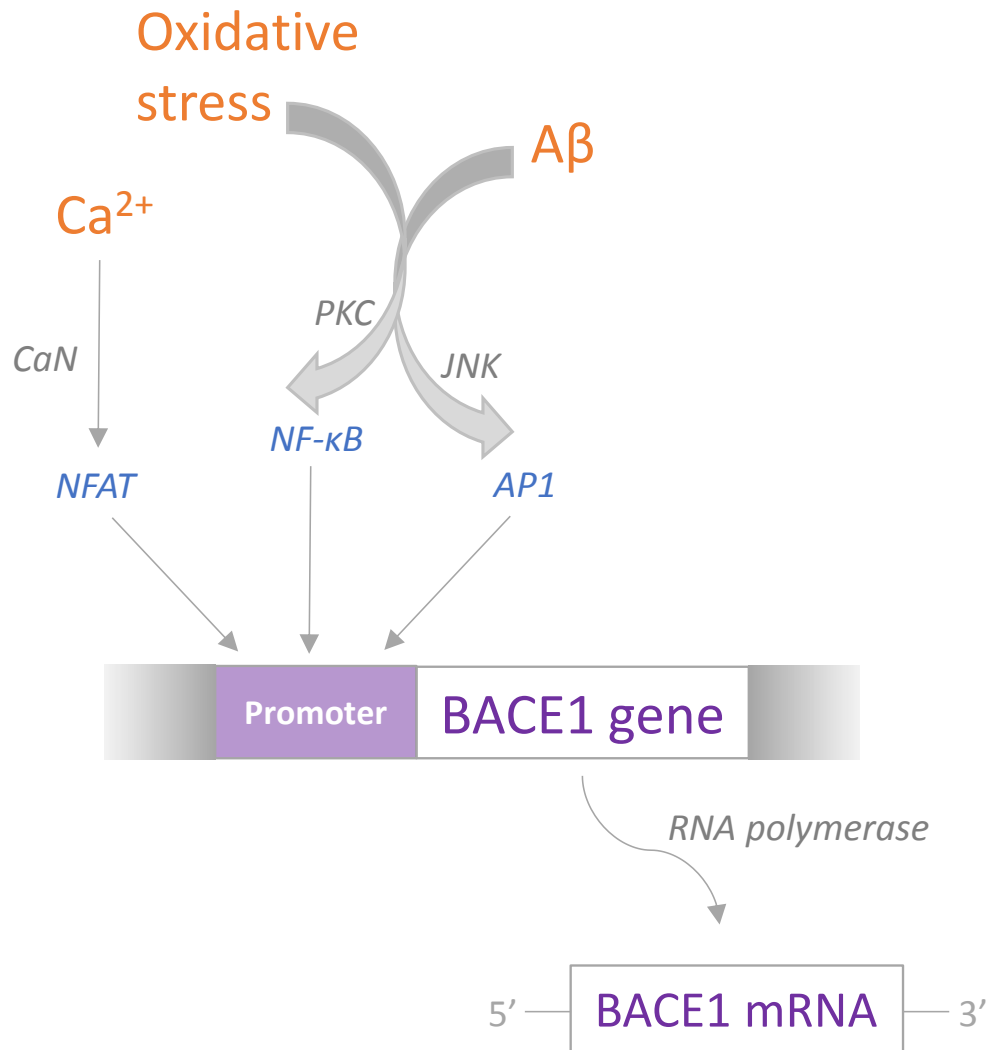


Figure 5.10 BACE1 protein levels can be enhanced by increased transcription. BACE1 promoter activity can be stimulated by oxidative stressors and A β , through protein kinase C (PKC) or Jun kinase (JNK) signalling, and by calcium, through calcineurin (CaN) signalling).

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hugely upregulated BACE1 transcription and protein levels measured in $\Delta 2$ -9 SH-SY5Ys in Chapter 4. Cells overexpressing Steap3 were also included, since Steap3 is an unrelated protein that has robust ferrireductase activity. The high production of hydrogen peroxide in Steap3 shows that ferrireductase activity could potentially contribute to the oxidative stress measured in α -syn overexpressing cells.

5.4.4 Could BACE1 expression be upregulated by transcription factors NF- κ B or AP-1, in α -syn cells?

Since there is evidence of elevated oxidative stress in α -syn SH-SY5Ys, oxidative stress could potentially mediate an effect on BACE1 transcription (Figure 5.10). In the literature, BACE1 promoter activity is enhanced by the oxidant H_2O_2 , in a mechanism involving the transcription factors NF- κ B and AP-1 (Tamagno et al. 2008; Marwarha et al. 2013). 27-hydroxycholesterol, 4-hydroxynonenal, and A β peptides, also activate the BACE1 promoter by similar means, although these are not exclusively oxidants (Buggia-Prevot et al. 2008; Marwarha et al. 2013; Tamagno et al. 2008). For example, 27-hydroxycholesterol increases ROS generation, but also appears to promote iron accumulation through a mechanism involving ER stress (Prasanthi et al. 2011). Iron has been suggested to increase BACE1 and ADAM10 transcription, APP translation, and C99 and C83 APP CTFs in cell culture (Kim & Yoo 2013; Guo et al. 2014). No evidence of higher BACE1 promoter activation was found in Chapter 4, in wildtype α -syn SH-SY5Ys with a luciferase reporter. Therefore, promoter upregulation of BACE1 is not evident in α -syn SH-SY5Ys. A specific contribution of NF- κ B and AP-1 to BACE1 protein levels were investigated in the current chapter. NF- κ B and JNK-1 inhibitors had been shown in the literature to suppress BACE1 expression, but unexpectedly augmented BACE1 protein levels in α -syn SH-SY5Ys. The contribution of A β to BACE1 levels was additionally assessed using a γ -secretase inhibitor, and this potentiated BACE1 expression in α -syn and empty vector cells. According to the literature, A β 42 activates NF- κ B and JNK-1 (Tamagno et al. 2008). Jo et al. (2010) found that γ -secretase inhibition reduces BACE1 expression in SH-SY5Ys, but this was in the context of acute oxidative stress stimuli (Jo et al. 2010). Two published studies have found that NF- κ B represses the BACE1 promoter in neuronal cells under physiological conditions, whereas pathological levels of A β switch NF- κ B into being stimulatory (Chami et al. 2012; Bourne et al. 2007). In light of this, the effect of NF- κ B inhibition in this chapter makes more sense. Clearly, WT α -syn SH-SY5Ys do not experience enough oxidative stress or A β production for the switch in NF- κ B activity to occur, and NF- κ B remains repressive. With this biphasic model of NF- κ B, the BACE1 promoter study (Chapter 4) can be re-interpreted. WT α -syn SH-SY5Ys had reduced BACE1 promoter activity compared with the empty vector, potentially as a

consequence of repressive NF- κ B activity. In contrast Δ 2-9 α -syn SH-SY5Ys had increased BACE1 promoter activity, perhaps indicating that NF- κ B is switched to stimulatory mode in these cells, which had particularly high β -amyloid production (Chapter 3). More research would be needed to test these theories. Regardless, the current data suggests that oxidative stress does not upregulate BACE1 transcription in α -syn cells.

5.4.5 Is there increased activation of eIF2 α phosphorylation in α -syn cells, and could this be upregulating BACE1 translation?

BACE1 expression can be regulated by translational de-repression, which occurs when cell stress activates the phosphorylation of eIF2 α , illustrated in Figure 5.11. BACE1 expression appears to be upregulated post-transcriptionally in α -syn SH-SY5Ys, so it was hypothesised that translational de-repression is involved. α -Syn expression increased levels of phosphorylated eIF2 α in SH-SY5Ys, creating permissive conditions for BACE1 translation. Although prone to noise, the average increase was almost a doubling of phosphorylated eIF2 α , as a ratio of total eIF2 α . Interestingly the Δ 2-9 mutation, which had the highest BACE1 protein levels in Chapter 4, significantly enhanced eIF2 α phosphorylation relative to the wildtype. Yet correlation does not necessarily mean causation, so levels of eIF2 α phosphorylation were additionally manipulated pharmacologically in α -syn and empty vector cells. Salubrinal reduces the targeting of phosphatase to eIF2 α , so phosphorylated eIF2 α should accumulate, highlighting any differences in basal eIF2 kinase activity between the cell lines. Accumulation of phosphorylated eIF2 α was weak, but both cell lines experienced significantly upregulated BACE1 protein. Overall there was not a clear difference in the response of α -syn cells to salubrinal, i.e. the basal eIF2 kinase activity. Since basal eIF2 α phosphorylation rates seemed similar, eIF2 α phosphorylation was subsequently stimulated to see whether α -syn cells were more sensitive to stress-mediated eIF2 kinase induction. Specifically, the response of eIF2 kinase PERK to stimulation by tunicamycin was tested in α -syn and empty vector cells. Tunicamycin reduces N-glycosylation of proteins in the ER, which prevents many proteins from folding adequately and leaving the ER (Bassik & Kampmann 2011). Since α -syn overexpression is thought to impair ER-Golgi transition (Cooper et al. 2006; Oaks et al. 2013; Thayanidhi et al. 2010), it was predicted that the cells would be particularly sensitive to tunicamycin-induced ER stress. However, the response of α -syn cells to tunicamycin was similar to control cells, with respect to eIF2 α phosphorylation as a biomarker of stress. It is thus not clear that the α -syn cells have a specific sensitivity to ER stress. Another mechanism must be responsible for the enhanced eIF2 α phosphorylation in α -syn SH-SY5Ys, perhaps independent of the ER.

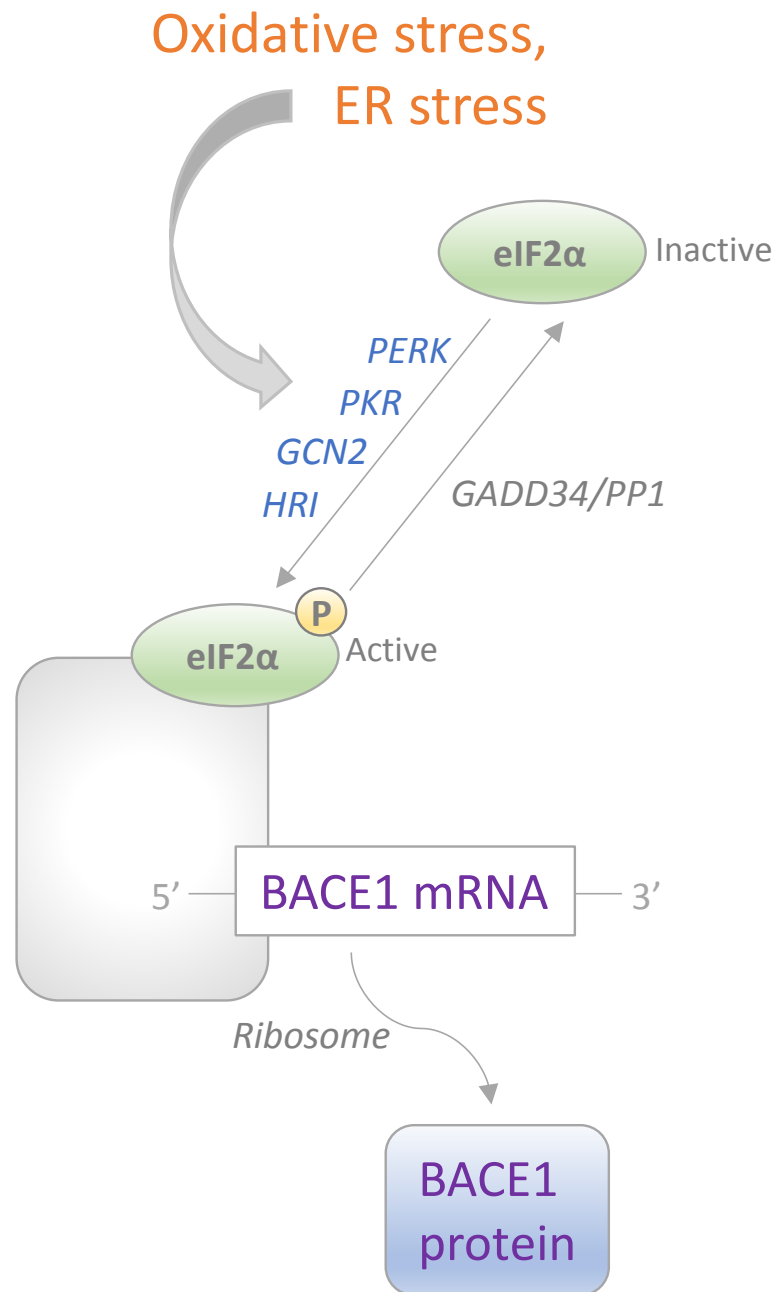


Figure 5.11 BACE1 protein levels can be enhanced by translational de-repression. BACE1 translation is stimulated by stress-mediated activation of eIF2 kinases, PERK, PKR, GCN2, and HRI, which phosphorylate eIF2 α . A GADD34/PP1 complex constitutively dephosphorylates eIF2 α .

5.4.6 Does *PKR* mediate translational upregulation of *BACE1* in response to oxidative stress in α -syn cells?

It is not yet clear whether a particular eIF2 kinase is upregulated in α -syn SH-SY5Ys. As discussed, PERK was investigated indirectly by observing the effect of tunicamycin on cell eIF2 α phosphorylation. Another eIF2 kinase of interest is PKR, which is activated by oxidative stress, but also ER stress and calcium stress (Marchal et al. 2014). To judge the potential role of PKR, a pharmacological inhibitor was used on α -syn and empty vector SH-SY5Ys. Relatively low concentrations of PKR inhibitor caused strong upregulation of eIF2 α phosphorylation in both lines, rather than the predicted reduction. The result suggests compensatory over-activation of another eIF2 kinase, a phenomenon previously described in the literature. Devi & Ohno reduced GCN2 expression by genetic means in mice, and found that PERK became over-activated, causing BACE1 expression to increase (Devi & Ohno 2013). One would expect BACE1 expression to follow suit. Yet PKR inhibitor treatment did not increase BACE1 protein levels, and a slight reduction was detected. The apparent uncoupling of BACE1 expression from eIF2 α phosphorylation may indicate that PKR promotes BACE1 expression by a different route, independent from its activity as an eIF2 kinase. PKR has many functions in signal transduction, including the activation of NF- κ B and inactivation of p53, so the effects of inhibition are manifold (Baltzis et al. 2007; Marchal et al. 2014). Pharmacological inhibition of PKR could allow active p53 to accumulate, leading to strong transcriptional repression of BACE1 (Singh & Pati 2015).

5.4.7 New hypotheses

To summarise, a couple of potential mechanisms for α -syn-induced BACE1 expression have been rendered improbable by the data gathered. It is unlikely that intracellular calcium is directly involved, or that the effect on BACE1 is transcriptionally regulated through NF- κ B or AP-1. Other putative mechanisms, translational upregulation and impaired degradation, remain viable. In future it would be worthwhile investigating further: (i) eIF2 α -mediated upregulation of BACE1 translation, (ii) decreased BACE1 targeting to lysosomes. The new hypotheses are displayed in Figure 5.12.

Elevated eIF2 α phosphorylation in α -syn SH-SY5Ys was observed, and could have a role in BACE1 expression. However, induced eIF2 α phosphorylation did not consistently lead to enhanced BACE1 protein levels, suggesting that another layer of regulation, perhaps involving BACE1 degradation, is at play. Post-translational effects have not been fully investigated, and there is some support for changes to BACE1 degradation in α -syn SH-SY5Ys. The effect is likely to be specific to BACE1 or a sub-set of proteins, and could involve reduced proteasomal

CHAPTER 5: POTENTIAL MECHANISMS UNDERLYING THE EFFECT OF α -SYN ON BACE1

or autophagic degradation. A global impairment of proteasomal degradation or autophagy ought to elevate levels of many other proteins, and decrease the fitness of the cell population, neither of which appears to be the case. Specific changes to BACE1 proteasomal degradation may be mediated by the E3 ligase CHIP, known to target BACE1 to the proteasome (Singh & Pati 2015). Alternatively, reduced lysosomal degradation of BACE1 may be a result of less GGA3, which specifically targets BACE1 to the lysosome (Kang et al. 2012).

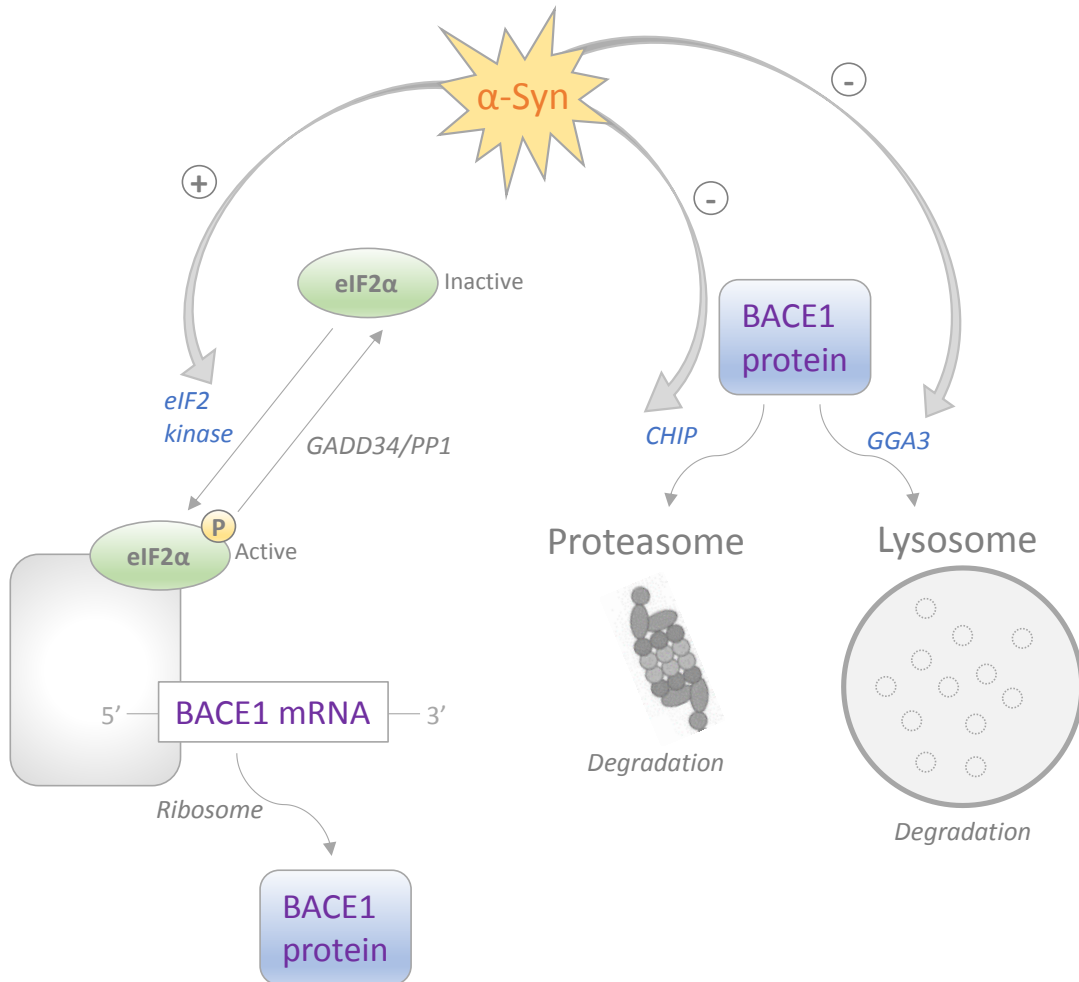


Figure 5.12 New hypotheses for future investigation of the effect of α -syn upon BACE1.

Elevated phosphorylation of eIF2 α in WT α -syn SH-SY5Ys suggests translational upregulation of BACE1. A mechanism involving translational upregulation of BACE1 would be supported by identification of the eIF2 kinase responsible, through genetic deletion of individual eIF2 kinases and acute α -syn overexpression. Impaired BACE1 degradation is implied by its over-accumulation in WT α -syn SH-SY5Ys treated with lysosome/proteasome inhibitors. The mechanism is speculated to involve reduced recruitment of GGA3 (lysosome) or CHIP (proteasome).

CHAPTER 6: CONCLUSIONS AND FURTHER WORK

6.1 Introduction

The major findings from this thesis can be summarised as follows. Chapter 3 established that the overexpression of α -syn in neuronal cell lines increases β - γ -secretase-mediated metabolism of APP, resulting in greater secretion of β -amyloid. Furthermore the effect appears to be potentiated by α -syn N-terminal mutations. Data in Chapter 4, on the expression and activity of secretase enzymes, showed changes to both BACE1 and ADAM10 expression in response to α -syn, but no apparent alteration to γ -secretase activity. Total protein levels of BACE1 were elevated, and ADAM10 reduced, but mature ADAM10 was enriched in the plasma membrane where it is reputedly more active. BACE1 levels correlated with α -syn expression in several models, and some exploration of the potential cell mechanisms connecting BACE1 to α -syn were outlined in Chapter 5. The results indicated that WT α -syn overexpressing cells exhibit biomarkers of cell stress, i.e. hydrogen peroxide production, and eIF2 α phosphorylation. BACE1 levels in these cells were also particularly sensitive to lysosome inhibition.

The effects of α -syn on various components of APP metabolism have been described in the individual chapter discussions. The purpose of this final discussion is to highlight other areas of research to which this thesis contributes.

6.2 α -Syn and APP in normal cell physiology

6.2.1 *α -Syn and APP are connected by intracellular processes*

The findings of this thesis support an intracellular connection of α -syn with APP processing. Fragments of α -syn and APP protein were demonstrated in 1993 to co-localise in amyloid plaques (Uéda et al. 1993), and have since been shown to co-aggregate *in vitro* (Ono et al. 2012; Tsigelny et al. 2008; Choi et al. 2015). Majd et al. showed that α -syn aggregates applied to primary neurons increased β -amyloid secretion (Majd et al. 2013). However, this may be the first time that a potential intracellular link between the proteins has been explored in any detail.

Until recently, α -syn and APP were believed to physiologically localise to completely separate membranes in a neuron, and interact only as a result of pathology (Marsh & Blurton-Jones 2012; Mandal et al. 2006). APP localises to post-synaptic membranes, whereas membrane-bound α -syn may predominantly localise to pre-synaptic vesicles (Maroteaux et al. 1988; Hoey et al. 2009). Cytosolic α -syn is distributed throughout the cell, but is thought to be unfolded and ‘inactive’ in this form. Yet APP and the β -secretase were discovered to localise to pre-synaptic vesicles and the pre-synaptic membrane (Groemer et al. 2011; Del Prete et al. 2014; Laßek et al. 2013). Similarly α -syn has been found at a number of other membranes, including ER and mitochondria, and alters the secretory pathway when over-expressed in several cell models (Snead & Eliezer 2014; Oaks et al. 2013; Thayanidhi et al. 2010).

The work detailed in this thesis confirms that distinct subcellular localisation does not prevent intracellular metabolic interactions between α -syn and APP metabolism. One limitation of this work is the simple cell model. Undifferentiated neuronal precursors were used, allowing ease of genetic manipulation, but with less well-defined postsynaptic and presynaptic compartments. Future work could study β -amyloid production from α -syn transgenic primary neurons, or differentiated iPSCs of an SNCA triplication patient (Devine et al. 2011).

6.2.2 N-terminal mutations of α -syn appear to cause a gain of function

A notable feature of the effect of α -syn on APP metabolism is that it was strongly potentiated by truncations within the N-terminus of the protein (Δ 2-9 and Δ NAC), and disease-associated point mutations had only a small effect. Strikingly, truncation of the extreme N-terminus or NAC domain are both known to inhibit aggregation of α -syn (Wang et al. 2010; Periquet et al. 2007). Toxic α -syn aggregates are therefore unlikely to be involved in the enhanced amyloidogenic processing of APP in α -syn cells. It would be natural to look to the physiological function of α -syn for answers.

α -Syn has two major properties that may be involved in its physiological function. Firstly, α -syn binds membranes with an N-terminal amphipathic helix (residues 1-95). The interaction of α -syn with membranes appears to regulate membrane tubulation and vesicle fusion events (Kamp et al. 2010; Lai et al. 2014; Jao et al. 2008; Varkey et al. 2010). Overexpression of WT and mutant α -syn could have a variety of different effects on cell membranes, a few of which are suggested by the literature. At the ER, WT α -syn maintains contacts with mitochondria, whereas A53T mutant α -syn reduces ER-mitochondrial contacts and induces mitochondrial fragmentation (Guardia-Laguarta et al. 2014). At the Golgi, over-expressed WT α -syn impedes docking and fusion of ER vesicles, potentially causing ER stress as well as delaying the

secretory pathway (Thayanidhi et al. 2010; Oaks et al. 2013; Gitler et al. 2008; Bellucci et al. 2011). In this role, the A53T mutation appears to exacerbate the ER-Golgi transport block, and exacerbates ER stress (Thayanidhi et al. 2010). At synaptic vesicles, WT α -syn promotes the assembly of exocytic machinery, and this effect is not altered by disease-associated point mutations such as A53T (Burré et al. 2012).

The effect of truncations Δ 2-9 and Δ NAC on the membrane-binding of the N-terminus could be subtle. The affinity of Δ NAC α -syn for membranes has not been studied, but some literature exists on Δ 2-9 suggesting only a small reduction in α -syn membrane binding, if any (Burré et al. 2012; Vamvaca et al. 2011; Wang et al. 2010). In Chapter 3 of this thesis a uniform cytosolic stain for α -syn was observed in Δ 2-9 SH-SY5Ys, suggesting reduced plasma membrane localisation. Without further evidence from western blotting, the result is equivocal. Overall, it seems likely that the N-terminal mutations had similar subcellular localisation to WT α -syn. This is supported in the literature by the normal synaptic targeting of α -syn, and normal α -syn-induced synaptic SNARE complex assembly in cells overexpressing Δ 2-9, A53T, or E46K (Burré et al. 2012). A potential model to explain the gain of APP amyloidogenic processing when the α -syn N-terminus is mutated, is a scenario where reduced negative regulation of α -syn occurs, e.g. reduced lysosomal targeting. This would increase or prolong α -syn activity, which could lead to greater ER-Golgi block and ER stress, or could have consequences for the co-localisation/stability of APP and the secretases.

Another known property of α -syn is its ability to reduce iron (Davies, Moualla, et al. 2011). This could potentially mediate the elevated levels of ROS production in α -syn SH-SY5Ys (Chapter 5). ROS stimulates the expression and activity of β - and γ -secretases on APP, so there is a well-understood link between ROS and APP metabolism (Tamagno et al. 2008; Jo et al. 2010). Unpublished work from our laboratory suggest that the effect of over-expressed α -syn upon β -amyloid production can be mitigated by treatment with an iron chelator, deferoxamine. It has been previously established that WT α -syn SH-SY5Ys contain higher than usual levels of reduced iron (Davies, Moualla, et al. 2011). Iron has been shown to increase the amyloidogenic processing of APP in an AD mouse model, and deferoxamine both inhibits β -amyloid accumulation and improves memory retention (Guo et al. 2013). Furthermore, deferoxamine reduces the sensitivity of A30P α -syn expressing M17 cells towards oxidative stress (Liddell et al. 2013). This supports a potential involvement of iron in β -amyloid production, via oxidative stress. Given that Δ 2-9, Δ NAC, and the disease-associated point mutations have high amyloidogenic processing of APP, one would predict that these enhance iron reduction in cells. Δ 2-9 protein also appeared to potentiate cell ROS production in

Chapter 5. So far, published (Davies, Moualla, et al. 2011) and unpublished data is inconclusive on whether these mutations increase iron reduction.

6.2.3 Perspective on the role of α -Syn toxic oligomers in cell dysfunction

In synucleinopathies, α -syn aggregation is widely regarded to be a key driver of cell dysfunction. Increased expression of wildtype α -syn is sufficient to induce α -syn aggregation in some models. When wildtype and disease-associated point mutations of α -syn are over-expressed in mice, the same changes to gene expression are seen, suggesting a shared pathophysiology (Miller et al. 2007). The common denominator is generally interpreted to be ‘toxic oligomers’ of α -syn, stabilised by molecular crowding or certain point mutations (Lashuel et al. 2013).

The stable cell lines used in this thesis have been selected to survive and proliferate with elevated α -syn expression. Yet the WT and mutant α -syn SH-SY5Ys exhibited biomarkers of cell stress, namely enhanced ROS production and phosphorylation of eIF2 α . Expression of mutant α -syn forms that are believed to be incapable of forming β -rich α -syn oligomers, Δ NAC and Δ 2-9, did not appear to reduce β -amyloid production or cell stress. In fact, these cells had higher levels of APP amyloidogenic processing than WT α -syn cells. The evidence does not support a leading role for α -syn toxic oligomeric species. It is likely that over-expressed wildtype α -syn acts on APP metabolism through an oligomer-independent mechanism. Potentially, APP metabolism could form a novel connection between α -syn function and disease.

There exists a great diversity of shapes and sizes of α -syn ‘toxic oligomer’ species, which exert numerous changes to cell signalling, metabolism and transport (Roberts & Brown 2015; Lashuel et al. 2013; Plotegher et al. 2014). The only factor apparently connecting these toxic oligomers is their β -rich structure, which is also shared with toxic oligomers of other proteins. In several unrelated amyloid-forming proteins, including A β 42, the toxicity of oligomers appears correlated with the exposure of hydrophobic residues, revealed by ANS binding. Additionally, particular structural epitopes in several unrelated toxic oligomers are recognised by ‘oligomer-specific’ antibodies (Bolognesi et al. 2010; Yoshiike et al. 2007; Kaye et al. 2003). Yet some of the biological effects of α -syn oligomers are not shared by others. For example, α -syn oligomers activate the IRE1 branch of the unfolded protein response, but PrP₁₀₆₋₁₂₆ and ABri₁₋₃₄ oligomers do not (Castillo-Carranza et al. 2012). What is unique about α -syn toxicity? Part of the answer may be that α -syn oligomers act by disrupting the physiological function of α -syn. Metastable α -syn aggregates may bind the ‘normal’ protein binding partners of α -syn, or sequester α -syn itself (Lashuel et al. 2013). This possibility has

not been explored extensively in the literature, but connections have been made between α -syn function and disease. For example, both monomers and large oligomers of α -syn were shown to bind synaptobrevin-2 in SNARE. Yet only large oligomers, and not monomers, impaired SNARE-mediated lipid mixing *in vitro*, and reduced exocytosis in PC12 cells (Choi et al. 2013). Another example of α -syn function-meeting-pathology is the putative role of α -syn in maintaining ER-mitochondrial contacts. The A53T α -syn disease mutant protein inhibits ER-mitochondrial contacts, and they can be rescued by increased expression of the wildtype protein (Guardia-Laguarta et al. 2014). There is therefore a precedent for A53T, and other disease-mutants, to exert an effect on APP metabolism by disturbing α -syn function.

6.2.4 APP metabolism is proposed be an evolved mechanism to cope with cell stress

Secretase-mediated processing of APP undergoes subtle and complex regulation. The expression, turnover, and subcellular localisation of all three secretase enzymes and APP are critical to the outcome of processing. It is difficult to fully explain the altered APP processing in α -syn cells without prolonged and detailed study, yet it is clear that expression of BACE1 and ADAM10 are altered, which is likely to contribute. BACE1 protein levels were enhanced, and ADAM10 protein appeared to have increased retention at the cell surface, suggesting an increase in β -secretase and α -secretase activity. An interesting question to ask is whether APP processing serves as an adaptive response to α -syn overexpression. To understand why APP processing could be a homeostatic mechanism, one needs to consider the physiological functions of amyloidogenic and non-amyloidogenic processing of APP in a cell.

Amyloidogenic and non-amyloidogenic processing are constitutive in neuronal cells, but are upregulated or downregulated by changes to secretase expression and activity. Broadly-speaking, BACE1 expression is upregulated in response to cell stresses, e.g. high intracellular ROS or calcium, ischaemic injury (Zhang et al. 2007; Tamagno et al. 2008; Cho et al. 2008). ADAM10 targeting to the plasma membrane is upregulated by neuronal activity, and this increases α -secretase processing of APP. PKC signalling from muscarinic receptors mediates the effect, which promotes binding of TGN-localised ADAM10 to a plasma membrane-targeting adaptor protein, SAP97 (synapse-associated protein 97) (Saraceno et al. 2014; Marcello et al. 2007).

Several APP fragments result from secretase-mediated processing of APP, potentially with different functions. Researchers looking for differences in the activity of β -cleavage fragments versus α -cleavage fragments have had limited success. The ectodomains sAPP α and sAPP β appear to both induce neural differentiation and proliferation (Freude et al. 2011; Lazarov & Demars 2012). Interestingly a new role in cholesterol regulation has been defined for the

ectodomain fragments, where they have distinct roles. sAPP α increases cholesterol biosynthesis, whereas sAPP β decreases cholesterol production, in a mechanism involving the transcription factor SREBP2 (sterol regulatory element-binding protein-2). The C-terminal fragments C99 (β -CTF) over C83 (α -CTF): ShcA and Pin1 (Repetto et al. 2004; Akiyama et al. 2005). ShcA activates MAPK signalling, and Pin1 is a prolyl isomerase that may recruit γ -secretase, or other adaptor proteins (van der Kant & Goldstein 2015). Overall it appears that β -cleavage and α -cleavage fragments have similar functions and little specialisation.

Even A β , the small peptide products of C99 cleavage by γ -secretase, are thought to have a function. Although associated with cytotoxicity, studies using a range of A β doses have revealed that it has the property of ‘hormesis’. Hormesis is where a factor has increasingly beneficial effects at low doses, but becomes less beneficial and even harmful at high doses. This can be thought of as an ‘inverted-U’ dose-response relationship. Hormesis is ubiquitous in drugs that are used to improve memory. The comparison is apt as, perhaps surprisingly, A β has many features in common with these drugs, illustrated in Figure 6.1 (Morley & Farr 2012; Puzzo & Arancio 2013). At picomolar concentrations, A β enhances synaptic plasticity and memory (Puzzo & Arancio 2013). Picomolar levels of A β stimulates the release of excitatory neurotransmitters from synapses, and also increase the activation of α 7- nicotinic acetylcholine receptors. Conversely, higher doses of A β are inhibitory to neurotransmitter release (Abramov et al. 2009; Mura et al. 2012). A β is both neurotrophic, in low doses, and neurodegenerative in high doses (Yankner et al. 1990). Additionally, at low concentrations A β acts as an antioxidant, binding to free copper, iron and zinc, and quenching free radicals (Nadal et al. 2008; Baruch-Suchodolsky & Fischer 2009; Kontush et al. 2001; Morley & Farr 2012; Pedersen et al. 2016). Higher levels of A β result in aggregation, the early stages appearing to produce a burst of hydrogen peroxide, and leading to oxidative damage in cell models (Mark et al. 1997; Tabner et al. 2005). The outcome of enhancing β -/ γ -secretase processing of APP therefore may depend on both baseline levels of A β and the degree of the increase.

How does this relate to the response of cells to α -syn overexpression? It is possible that the resulting increase in β -/ γ -secretase processing of APP is simply a side-effect of overexpressing a protein that interferes with the secretory and endocytic pathways. Alternatively, increased β -/ γ -secretase processing of APP may be an adaptive response to cell stress caused by the α -syn. Secreted A β levels were only doubled by the α -syn overexpression, and likely to be in the range at which A β is antioxidant and neuroprotective. Indirectly supporting this, the use of a γ -secretase inhibitor to suppress A β production in α -syn overexpressing cells had the effect

of enhancing BACE1 expression. The effect on BACE1 indicates a feedback pathway to restore amyloidogenic processing, suggesting that this serves a useful function.

6.3 α -Syn and APP in neurodegenerative disease

6.3.1 *Lewy Body dementias*

The novel finding that increased α -syn increases the production of A β could shed some light on the relationship between PD and AD. A spectrum of Lewy Body dementias (PDD and DLB) is thought to exist, shown in Figure 6.2. Both PDD and DLB are classified as synucleinopathies but have some of the pathophysiological features of AD, including the occurrence of amyloid plaques and neurofibrillary tangles (Berg et al. 2014). As high as 80% of PD patients develop dementia within a decade of PD diagnosis (Emre et al. 2007). Although dementia is common in old age, α -syn accumulation is likely to contribute. Increased expression of wildtype α -syn occurs in patients with SNCA duplication; all known cases of SNCA duplication were diagnosed with DLB, rather than pure PD. Autopsy reveals widespread Lewy bodies in the neocortex, which is known to correlate strongly with cognitive impairment (Konno et al. 2016). Cortical Lewy bodies may be important to the development of dementia in PD patients, but researchers are intrigued by the potential involvement of concomitant amyloid plaques and neurofibrillary tangles, the key features of AD pathology.

New research is uncovering the significance of A β pathology to development of dementia in PD. Several studies have used cerebrospinal fluid (CSF) biomarkers of α -syn and A β pathology, to study cognitive decline in live patients with early stages of PD. Decreasing levels of CSF A β 42 are an established predictive biomarker for cognitive decline in AD, and linked to increasing amyloid plaque deposition (McKhann et al. 2011). Similarly, studies of early PD show that a decrease in CSF A β 42 appears to immediately precede cognitive impairments, and has predictive power for PD patients that develop dementia (Stav et al. 2015; Vranová et al. 2014; Hall et al. 2015; Skogseth et al. 2015; Terrelonge et al. 2015; Alves et al. 2014). PET imaging of patients using the Pittsburgh compound B (PiB) can also be performed to detect areas of A β insoluble aggregates in the brain, and agrees with the CSF data (Petrou et al. 2015). Furthermore, the idea of a PD-AD disease spectrum is supported by CSF A β 42 measurements and PiB imaging, ranking on average: PD<PDD<DLB<AD (Vranová et al. 2014; Petrou et al. 2015).

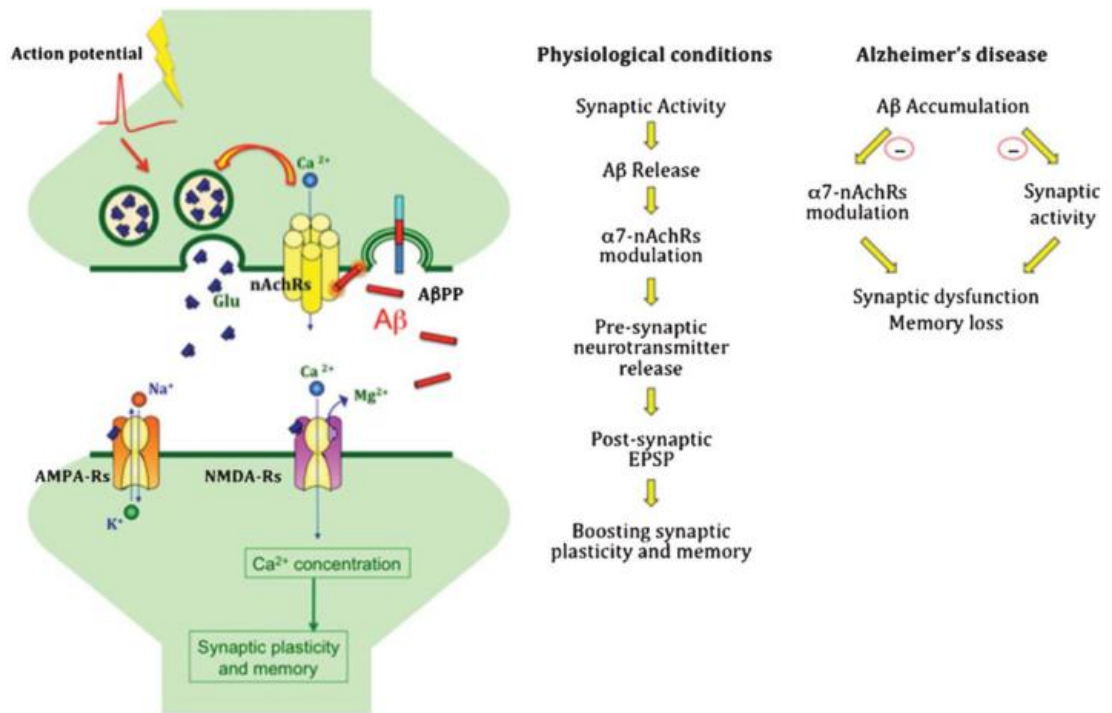


Figure 6.1 Proposed physiological and pathological roles of Aβ in synaptic plasticity and memory. Taken from (Puzzo & Arancio 2013).

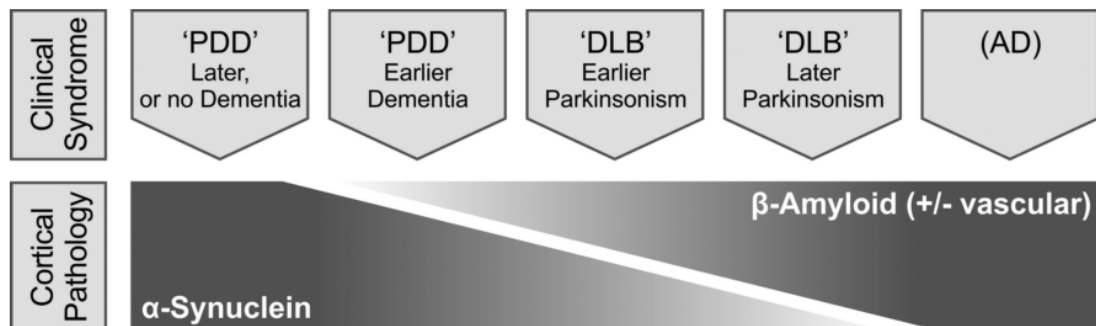


Figure 6.2 The PD-AD spectrum. A simplified model of the way that clinical presentation may relate to cortical α-syn and Aβ pathology. Taken from (Berg et al. 2014).

Although amyloid plaques in the striatum contribute to cognitive impairment, amyloid deposition does not appear to correlate with the degeneration of nigrostriatal neurons in PD (Chiaravalloti et al. 2014; Shah et al. 2015). This suggests that A β pathology is secondary to α -syn pathology in PD. Of particular interest, some studies find that CSF α -syn levels are low in PD patients with early cognitive impairment, and also closely correlate with CSF A β 42 (Skogseth et al. 2015; Buddhala et al. 2015). The implication of this is that changes to α -syn pathology occur at the same time as A β deposition, signifying a relationship rather than mere coincidence. CSF α -syn is not prognostic of dementia to the same degree as CSF A β 42, but is not an extensively validated biomarker of brain α -syn pathology (Parnetti et al. 2014). One may surmise from the current evidence that both α -syn and A β contribute to the development of dementia in PD.

The question remains: what relationship do α -syn and A β have in the brains of PD patients? Three hypotheses will be discussed, also illustrated in Figure 6.3, which are not mutually exclusive.

- (A) **α -Syn and A β pathology directly enhance one another's aggregation.** A direct effect of α -syn in 'templating' the assembly of A β 42 amyloid fibrils has been observed *in vitro*, and *vice versa* (Atsmon-Raz & Miller 2015; Mandal et al. 2006; Ono et al. 2012; Masliah et al. 2001). This is known as 'cross-seeding'. However although the two proteins co-immunoprecipitate from AD/PD brains (Tsigelny et al. 2008), cross-seeding does not manifest in mouse models. In fact, α -syn may inhibit amyloid plaques in mice. In AD model mice, the overexpression of A30P α -syn appears to reduce A β deposition, and appeared to reduce the seeding activity of A β aggregates (Bachhuber et al. 2015). Conversely, knock-out of α -syn increases plaque load in AD mice (Kallhoff et al. 2007).
- (B) **A common upstream event**, perhaps an acute cellular insult, upregulates both α -syn and A β aggregation. Cells in ageing brains are less well-equipped to cope with disordered proteins such as α -syn and A β , due to an age-related decline in the activity of protein degradation pathways, molecular chaperones, and antioxidants. Support for this concept comes from studies of exposure to toxins that induce parkinsonism. For example, manganese exposure in primates causes accumulation of both intracellular α -syn aggregates and diffuse extracellular A β aggregates in the frontal cortex, associated with neurodegeneration (Verina et al. 2013). Nigrostriatal neurons were spared in this model, but PET imaging showed markedly reduced dopamine release in the striatum (Guilarte et al. 2008). Once α -syn and A β aggregation has been triggered, the pathology may become self-

sustaining through similar cell mechanisms. α -Syn and A β aggregates arise from accumulation of copper and iron, high ROS production, and impaired protein degradation, but also create a positive feedback effect on these same factors, reviewed in (Jomova et al. 2010; Malkus et al. 2009). Yet with this scenario it is difficult to explain why most synucleinopathy is absent of significant A β pathology, and why α -syn pathology is limited in AD (Berg et al. 2014).

(C) α -Syn aggregation could occur first, and over time promote A β deposition.

This thesis provides a mechanism by which this could occur, through the enhanced amyloidogenic processing of APP, although there is not yet any evidence that this *does* occur in disease. Pathological studies support that cognitive decline in PDD and many, but not all, cases of DLB are primarily driven by cortical α -syn pathology (Deramecourt et al. 2006). Indeed, in several studies amyloid pathology has been absent in most PDD brains, and is significantly more widespread in DLB (Petrout et al. 2015). Ruffman et al. found that DLB brains also have significantly higher levels of α -syn aggregates in the temporal and parietal lobes than PDD brains, and this correlates with a shorter time interval between motor and dementia symptoms (Ruffmann et al. 2015). Of course, there remains a question of why many PD patients do not develop A β accumulation and deposition, if this is promoted by α -syn. The answer could be that A β deposition develops in PD patients with a genetic predisposition. Some genetic component has been found to PDD and DLB, in a study of 174 patients that discovered rare missense variants of known AD or PD-associated genes (e.g. PSEN2, PARK2) in 6.8% of the cohort. Among the DLB patients there was significant prevalence of major AD risk allele ApoE ϵ 4, and in the PDD/ DLB cohort a greater frequency of variants of the PD risk gene glucocerebrosidase was detected (Meeus et al. 2012). However, the extent of genetic predisposition is uncertain.

6.3.2 Alzheimer's disease

Although this thesis has only explored one side of the relationship between α -syn and APP/ A β , it is possible that the relationship goes two ways, i.e. there is an effect of APP/ A β on α -syn. A two-way relationship may explain why some cases of DLB with late parkinsonism exhibit only limited α -syn pathology that may be secondary to A β . Support for this hypothesis arises from coincident pathology in AD.

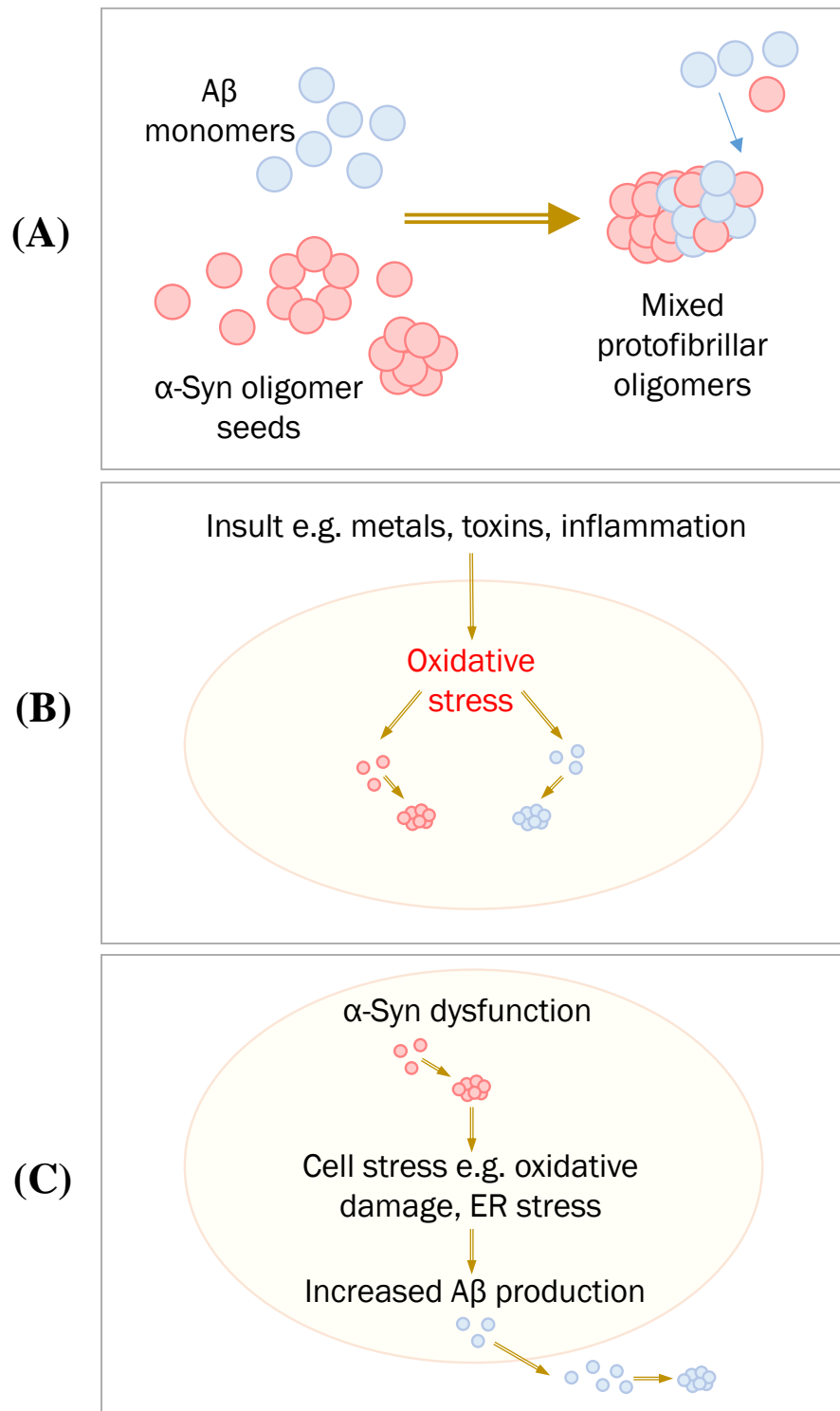


Figure 6.3 Three types of relationship between α -syn and A β that have been hypothesised to occur in neurodegenerative disease.

Lewy bodies are frequently widespread in AD brains, in the absence of parkinsonism, estimated to occur in ~25-40% of AD by the time of death (Jellinger 2003; Uchikado et al. 2006; Schneider et al. 2009). One study found Lewy bodies in 30% of sporadic AD brains and 27% of genetic (autosomal dominant) AD brains. The extent of Lewy body pathology was scored significantly greater in the sporadic cases compared with genetic AD (Ringman et al. 2016). Cell experiments also support a potentially indirect effect of A β on α -syn. Majd et al. found that recombinant A β 42 aggregates added to hippocampal neurons increased total intracellular levels of α -syn protein (Majd et al. 2013). Swirski et al. took a different approach, studying insoluble, S129-phosphorylated α -syn, which is scarce in healthy cells but predominates in Lewy bodies. Exposure of α -syn SH-SY5Ys to A β aggregates increased the proportion of insoluble α -syn that was phosphorylated at S129, which suggests an increase in α -syn fibril-formation (Swirski et al. 2014). Future exploration of this effect would be informative, and could complement studies of the effect of α -syn on APP.

6.4 Future work

This thesis has sketched out the rudiments of the effect of α -syn upon APP processing, and future work should focus on fleshing out some of the mechanistic detail. Primarily, I would like to ascertain the extent to which BACE1 controls α -syn-mediated β -amyloid production, for example with RNA-interference to reduce its activity. The endosomal co-localisation of BACE1 and APP in α -syn cell models should also be explored, since cell localisation is thought to have a major impact on APP processing. Increased BACE1/APP co-localisation could result from reductions in the lysosomal targeting and degradation of BACE1. Preliminary evidence suggested that BACE1 degradation in α -syn SH-SY5Ys was impaired, so exploring this could prove fruitful. A translational effect of BACE1 could also be confirmed with a BACE1 5'UTR luciferase reporter (ILL-Raga et al. 2011). Likely mechanistic connections between α -syn and BACE1 expression include the activation of eIF2 kinases by oxidative stress or ER stress. Perhaps overexpression of constitutively active GADD34, to repress eIF2 α phosphorylation, could be used to assess the contribution of BACE1 translational de-repression (Sadleir et al. 2014). Activation of individual eIF2 kinases, PKR, PERK, GCN2, and HRI, could be biochemically characterised. It may also be interesting to see whether the effect of α -syn on APP can be mitigated by approaches to decrease oxidative stress, such as antioxidants. α -Syn is known to impair secretory pathway trafficking in a way that is rescuable by Rab GTPase overexpression (Cooper et al. 2006; Dalfó et al. 2004), so studying the overexpression of specific Rab GTPases on α -syn-mediated β -amyloid production could be a fascinating tangent to the project. Indeed, the interaction of α -syn with

Rab8a has been shown to be dependent of α -syn S129 phosphorylation (Yin et al. 2014), which appeared to have a small impact on APP amyloidogenic processing in Chapter 3.

The intriguing role of S129 phosphorylation in the effect of α -syn on APP processing is indicative of an underlying structure-function relationship that could be further explored. It is still not clear what features of α -syn contribute to its effect on APP, particularly since none of the mutations studied significantly mitigated the effect. Perhaps significant reduction of α -syn membrane association would eliminate the effect of α -syn on APP. Perhaps removal of the C-terminus, a site for binding physiological interactors such as synaptobrevin-2 (Burré et al. 2010), would decrease APP amyloidogenic processing. It may also be interesting to see whether α -syn knock-down in cells has the opposite effect to overexpression.

6.5 Concluding remarks

Previous research into the potential interactions between α -syn and APP have focussed on aggregation, of α -syn and/or the A β peptide, to the detriment of investigating the underlying cell biology. The discovery that α -syn alters secretase-mediated APP processing in this thesis, and dissection of the underlying changes to secretase activity, has shown that the cell biology connecting these two proteins is interesting and worth further study. A precise mechanism is yet to be defined, despite attempts in this thesis to find it, but will surely prove to be enlightening. From the perspective of treating diseases such as DLB, where both α -syn and A β pathology appears within a short frame of time, understanding the connecting cell biology could be vital. If, for example, the A β production is a cellular response to a pre-existing synucleinopathy, then it may be unnecessary or even harmful to suppress A β production with β -secretase inhibitors. Ultimately, further development of this study may allow it to shape the future of therapeutics in synucleinopathy disease.

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